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THE ROLE OF MITOCHONDRIAL CREATINE KINASE AND HEXOKINASE IN CARDIOPROTECTIVE MECHANISMS INDUCED BY CHRONIC HYPOXIA

ÚLOHA MITOCHONDRIÁLNÍ KREATINKINÁZY A HEXOKINÁZY V MECHANISMECH KARDIOPROTEKTIVNÍHO PŮSOBENÍ CHRONICKÉ HYPOXIE

Ph.D. THESIS

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Declaration

I hereby declare that I completed this Ph.D. thesis independently, except where explicitly indicated otherwise. It documents my own work, carried out under the supervision of Dr. Jitka Žurmanová, Ph.D. Throughout, I have properly acknowledged and cited all sources used. Neither this thesis nor its substantial part has been submitted to obtain this or other academic degree.

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ABSTRACT IN ENGLISH

The ischemia-reperfusion (I/R) injury, which is a consequence of myocardial infarction, represents a major cause of death worldwide. One of the most effective cardioprotective interventions increasing the resistance of hearts to the I/R injury is the adaptation to a chronic hypoxia (CH). However, the molecular mechanisms of CH are still not well understood. The most important factors responsible for the I/R injury are reactive oxygen species (ROS) produced by complexes I and III within the mitochondrial electron transport chain. Potential candidates maintaining ROS at a low level are mitochondrial creatine kinase (mtCK) and two hexokinase isoforms (HK1 and HK2). These enzymes highly support the mitochondrial oxidative phosphorylation by increasing the availability of ADP for complex V of the respiratory chain. In addition, the HK binding to mitochondria inhibits binding of the pro-apoptotic protein BAX, thereby protecting cardiac cells against apoptosis. Besides the mitochondrial CK isoform, there are two cytosolic CK (CKM and CKB) present in cardiomyocytes that help to maintain energy homeostasis. Based on the known anatomical and physiological differences between the left (LV) and the right (RV) ventricles, the first study focused on the comparing ventricles in terms of the energy metabolism and the HK co-localization with mitochondria. Further, the level of activated AKT kinase, which facilitates interaction of HK2 with mitochondrial membrane, was determined. The results of this study indicate that the RV has a higher activity of aerobic glycolytic metabolism and may be able to respond faster and more powerfully to stressful stimuli than the LV. The results also suggest that AKT activation is a necessary but not a sufficient condition for the enhancement of the interaction of HK2 with mitochondria and that yet another mechanism may be involved. Next, this study aimed at the effect of the normobaric hypoxia on the CK and HK expressions and enzyme activities and the HK co-localization with mitochondria in both ventricles. Rats were adapted for 3 weeks to protective and non-protective regimens of 10% normobaric hypoxia. The results showed that the adaptation to the normobaric hypoxia leads to the activation of glycolysis and phosphocreatine (PCr)/CK system to maintain energy homeostasis under the reduced oxygen concentration. This may suggest that CK and HK can be involved in the

stimulation of the oxidative phosphorylation, which reduces the production of ROS. Although no differences were found between protective and non-protective phenotypes, it cannot be ruled out that CK and HK may play a role in the cardioprotective mechanisms induced by the normobaric hypoxia. Interestingly, HK1 and HK2 co-localizations with mitochondria remained unchanged in the LV as well as in the RV after adaptation of rats to a protective regimen, continuous normobaric hypoxia (CNH), suggesting a stabilization of the HK bond with mitochondria. Then, rats adapted to CNH were subjected to the I/R insult. The HK activity significantly increased in the CNH LV after the I/R insult, which can suggest that the HK can possibly participate in the establishment of the ischemia-resistant phenotype of chronically hypoxic hearts. Finally, the last objective of the study was to investigate the role of CK and HK enzymes in the LV and RV of rats adapted to a hypobaric hypoxia (7000 m). The hypobaric hypoxia represents a greater stress for the myocardium compared to the normobaric hypoxia. The results verified the response of energy metabolism to the reduced oxygen level independently on the degree of stress. In addition, the HK1 and HK2 co-localizations with mitochondria markedly increased in both ventricles after adaptation of rats to hypobaric hypoxia, which was confirmed by the immunofluorescence technique as well as by the fractionation and Western blot method. In conclusion, the protective mechanism of the HK, which lies in its binding with mitochondria, seems to be activated under the hypoxia which is marginal for the fatal cell damage, representing by present hypobaric model. This dissertation study provides a novel insight in the CK and HK function and co-operation under different hypoxic adaptations. And it also provides new information related to cardioprotective mechanisms of adaptation to hypobaric hypoxia, which includes increased binding of the HK with mitochondria. Targeting the HK binding with mitochondria thus represents a potential approach for future therapeutic uses.

ABSTRACT IN CZECH

Ischemicko-reperfúzní (I/R) poškození, které je důsledkem infarktu myokardu, představuje hlavní příčinu úmrtí po celém světě. Jedním z nejúčinnějších kardioprotektivních intervencí zvyšujících odolnost srdce k I/R poškození je adaptace na chronickou hypoxii (CH). Avšak molekulární mechanismy CH nejsou zcela objasněny. Mezi nejdůležitější faktory zodpovědné za I/R poškození patří reaktivní formy kyslíku (ROS) produkované komplexy I a III mitochondriálního elektronového transportního řetězce. Potenciálními kandidáty udržující ROS na nízké úrovni jsou mitochondriální kreatinkináza (mtCKs) a dvě isoformy hexokinázy (HK1 a HK2). Tyto enzymy významně podporují mitochondriální oxidativní fosforylaci tím, že zvýšují dostupnost ADP pro komplex V dýchacího řetězce. Navíc HK navázaná na mitochondrie inhibuje navázání pro-apoptotického proteinu BAX, čímž chrání srdeční buňky vůči apoptóze. Kromě mitochondriální isoformy CK jsou v kardiomyocytech také přítomny dvě cytosolické isoformy CK (CKM a CKB), které pomáhají udržovat energetickou homeostázu. Na základě známých anatomických a fyziologických rozdílů mezi levou (LV) a pravou (RV) komorou se první studie zaměřila na porovnání těchto komor z hlediska energetického metabolismu a ko-lokalizace isoforem HK s mitochondriemi. Také byla stanovena hladina aktivované formy AKT kinázy, která napomáhá interakci HK2 s mitochondriální membránou. Výsledky této studie naznačují, že RV má vyšší aktivitu aerobního glykolytického metabolismu a je tedy schopna reagovat rychleji a silněji na stresující podněty než LV. Dále bylo zjištěno, že aktivace AKT je nutnou, nikoli však postačující podmínkou pro stimulaci interakce HK2 s mitochondriemi a že je zde pravděpodobně zapojen ještě jiný mechanismus. Dalším cílem studie bylo stanovit vliv normobarické hypoxie na expresi a enzymatickou aktivitu CK a HK a ko-lokalizaci HK s mitochondriemi u obou komor. Potkani byli adaptováni po dobu 3 týdnů na protektivní a neprotektivní režimy 10% normobarické hypoxie. Výsledky ukázaly, že adaptace na normobarickou hypoxii vede k aktivaci glykolýzy a kreatinfosfát (PCr)/CK systému udržujícího energetickou homeostázu i za snížené koncentrace kyslíku. Dá se tedy usuzovat, že CK a HK by se mohly podílet na stimulaci oxidativní forforylace a tím na snížení produkce ROS. I když nebyly zjištěny žádné rozdíly mezi protektivními a

neprotektivními fenotypy, nelze vyloučit, že CK a HK mohou hrát roli v kardioprotektivních mechanismech vyvolaných adaptací na normobarickou hypoxii. Je zajímavé, že ko-lokalizace HK1 a HK2 s mitochondriemi zůstaly nezměněny v LV i v RV po adaptaci potkanů na protektivní režim, kontinuální normobarickou hypoxii (CNH), což svědčí o stabilizaci vazby HK s mitochondriemi. Následně byli potkani adaptovaní na CNH vystaveni I/R inzultu. Celková aktivita HK v CNH LV se výrazně zvýšila po I/R inzultu, což by mohlo vypovídat o zapojení HK v mechanismech ischemicko-rezistentního fenotypu u chronicky hypoxických srdcí. Posledním cílem této studie bylo určit roli CK a HK enyzymů v LV a RV potkanů adaptovaných na hypobarickou hypoxii (7000 m). Hypobarická hypoxie představuje vyšší stres na myokard ve srovnání s normobarickou hypoxií. Výsledky potvrdily reakci energetického metabolismu na sníženou koncentraci kyslíku nezávisle na míře stresu. Navíc byla pozorována zvýšená vazba HK1 a HK2 s mitochondriemi v obou komorách po adaptaci potkanů na hypobarickou hypoxii, která byla potvzena jak pomocí imunofluorescenčních technik, tak i pomocí frakcionace a metody Western blot. Závěrem lze říci, že protektivní mechanismus HK, který souvisí s mírou vazby tohoto enzymu na mitochondriích, se zdá být aktivován v hypoxii, která je na hranici poškození buněk, což představuje právě hypobarický model. Tato disertační práce poskytuje nový pohled na funkci CK a HK a jejich vzájemnou spolupráci v adaptaci na různé modely hypoxie. A zároveň přináší nové poznatky o mechanismu kardiprotektivního působení hypobarické hypoxie, který zahrnuje zvýšenou vazbu HK s mitochondriemi. Studium vazby HK s mitochondriemi tak představuje potenciální cestu pro budoucí terapeutické využití.

LIST OF ABBREVIATIONS

ADM	Adrenomedullin
ADP	Adenosine 5'diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AK1, 2, 3	Adenylate kinase 1, 2, 3
AMP	Adenosine 5'monosphate
AMPK	5'AMP-activated protein kinase
ANT	Adenine nucleotide translocase
AP1, 2	Activating protein 1, 2
ARNT	Aryl hydrocarbon nuclear translocator
ATP	Adenosine 5'triphosphate
BKCa channels	Ca^{2+}-activated K^{+} channels
BNIP3	Bcl-2/adenovirus E1B 19-kDa protein-Interacting Protein 3
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine 5'monophosphate
CAT	Catalase
cGMP	Cyclic guanosine 5'monophosphate
CH	Chronic hypoxia
CK	Creatine kinase
CKB	Cytoplasmic brain CK
CKM	Cytoplasmic muscle CK
CNH	Continuous normobaric hypoxia
CP	Crossing point
Cr	Creatine
-CoA	-Coenzyme A
COX	Cytochrome *c* oxidase
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
eNOS	Endothelial NO
E	specific PCR efficiency

EPO	Erythropoietin
ERK1/2	Extracellular signal-regulated kinase 1/2
FFA	Free fatty acid
FIH	Factor inhibiting HIF
F-2,6-P	Fructose-2,6-phosphate
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
G-6-P	Glucose-6-phosphate
GSK3	Glycogen synthase kinase 3
GTP	Guanosine 5'triphosphate
HIF1	Hypoxia inducible factor 1
HK	Hexokinase
HO1	Heme oxygenase 1
HRE	Hypoxia response element
HPRT1	hypoxanthine-guanine phosphoribosyltransferase 1
IGF1	Insulin-like growth factor 1
IH	Perfused hypoxic hearts subjected to stabilization and ischemia
IHH	Intermittent hypobaric hypoxia for 8 h/day with a single 16-h normoxic period per day
IN	Perfused normoxic hearts subjected to stabilization and ischemia
INH-8	Intermittent normobaric hypoxia for 8 h/day with a single 16-h normoxic period per day
INH-23	Intermittent normobaric hypoxia for 23 h/day with a single 1-h normoxic period per day
iNOS	Inducible NOS
I/R injury	Ischemia-reperfusion injury
IRH	Perfused hypoxic hearts subjected to stabilization, ischemia, and reperfusion
IRN	Perfused normoxic hearts subjected to stabilization, ischemia, and reperfusion

IP_3	Inositol-1,4,5-triphosphate
KH	Perfused hypoxic hearts subjected to stabilization
KN	Perfused normoxic hearts subjected to stabilization
LDH	Lactate dehydrogenase
L-NAME	N(omega)-nitro-L-arginine methyl ester
LV	Left ventricle
MAPKAP-2	mitogen-activated protein kinase-activated protein kinase 2
MCT1, 4	Monocarboxylate transporter 1,4
MEF	Myocyte enhancer factor
MPT pore	Mitochondrial permeability transition pore
mtCKs	Sarcomeric mitochondrial CK isoform
mtCKu	Ubiquitous mitochondrial CK
mTORC1, 2	mammalian target of rapamycin complex 1, 2
MyHCα, β	Myosin heavy chain α, β
N	Normoxia
NAD/NADH	Nicotinamide adenine dinucleotide
NAPDH	Nicotinamide adenine dinucleotide phosphate
NCX	Na^+/Ca^{2+} exchanger
NDPK-D	Nucleoside diphosphate kinase
NFκB	Nuclear factor kappa B
NMPK	Nucleoside monophosphate kinase
NO	Nitric oxide
NOS	Nitric oxide synthase
NR2F2	Nuclear receptor subfamily 2, group F, member 2
O-CK	Oxidized CK form
OXPHOS	Compartments of the mitochondrial respiratory chain and the ATP synthase
PARP-1	NAD^+ ADP-ribosyltransferase-1
PCr	Phosphocreatine
PCr/CK system	Phosphocreatine/Creatine kinase system
PDC	Pyruvate dehydrogenase kinase complex

PDE3B	Phosphodiesterase 3B
PDH	Pyruvate dehydrogenase
PDK1	Phosphoinositide-dependent kinase1
PFK1, 2	Phosphofructokinase1, 2
PGC1α, β	PPAR γ coactivator 1α, β
PGK	Phosphoglycerate kinase
PHDs	Prolylhydrolylases
Pi	Iorganic phosphate
PIC	Phosphate carrier
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3K/AKT	Phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT kinase
PIKfyve kinase	FYVE zinc finger domain kinase binding phosphatidylinositol 3-phosphate
PIP_3	Phosphatidylinositol (3,4,5) trisphosphate
PK	Pyruvate kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
p38 MAPK	p38 mitogen-activated protein kinase
pO_2	partial pressure of oxygen
PPARα, γ	peroxisome proliferator-activated receptor α, γ
R-CK	Reduced CK form
ROI	Regions of interest
ROS	Reactive oxygen species
RT	Room temperature
RV	Right ventricle
RyR	Ryanodine receptors
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	Sarcolemmal Ca^{2+} ATPase
SOD	Superoxide dismutase
TBS	Tris-buffered saline solution

TCA	Tricarboxylic acid cycle
TNFα	Tumor necrosis factor α
TTBS	TBS containing Tween 20
VDAC	Voltage dependent anion channel
VEGF	Vascular endothelial growth factor
WB	Western blot
WHF	World Heart Federation
WHO	World Health Organization

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LIST OF PUBLICATIONS

a) Dissertation related publications:

Waskova-Arnostova P, Kasparova D, Elsnicova B, Novotny J, Neckar J, Kolar F, Zurmanova J. Chronic hypoxia enhances expression and activity of mitochondrial creatine kinase and hexokinase in the rat ventricular myocardium. Cell Physiol Biochem. 2014;33(2):310-20. doi: 10.1159/000356671. Epub 2014 Jan 31.

IF = 3.415

Waskova-Arnostova P, Elsnicova B, Kasparova D, Sebesta O, Novotny J, Neckar J, Kolar F, Zurmanova J. Right-to-left ventricular differences in the expression of mitochondrial hexokinase and phosphorylation of Akt. Cell Physiol Biochem. 2013;31(1):66-79. doi: 10.1159/000343350. Epub 2013 Jan 22.

IF = 3.415

b) Other publications:

Radosinska J, Bacova B, Knezl V, Benova T, Zurmanova J, Soukup T, **Arnostova P**, Slezak J, Gonçalvesova E, Tribulova N. Dietary omega-3 fatty acids attenuate myocardial arrhythmogenic factors and propensity of the heart to lethal arrhythmias in a rodent model of human essential hypertension. J Hypertens. 2013;31(9):1876-85. doi: 10.1097/HJH.0b013e328362215d.

IF = 3.806

Arnostova P, Jedelsky PL, Soukup T, Zurmanova J. Electrophoretic mobility of cardiac myosin heavy chain isoforms revisited: application of MALDI TOF/TOF analysis. J Biomed Biotechnol. 2011;2011:634253. doi: 10.1155/2011/634253. Epub 2011 Nov 30.

IF = 2.88

1. INTRODUCTION

1.1 Energy metabolism of the heart

The heart is a unique highly dynamic organ that supplies oxygen and necessary nutrients to the other tissues throughout the life. Maintaining the continuous contraction, ionic homeostasis, and cell signaling requires large amounts of energy and unconditional continuous supply thereof. The primary source of energy for the heart is represented by the adenosine 5'triphosphate (ATP) molecule. As the heart is a primarily aerobic organ containing a large number of mitochondria, almost all (~ 95%) of the generated ATP comes from the oxidative phosphorylation. The rate of ATP production is closely linked to the rate of ATP consumption so that the level of ATP remains constant even with an intense cardiac workload (reviewed in Stanley et al., 2005). The mammalian heart preferentially utilizes free fatty acids (FFA) as the major energy substrates, because their complete oxidation is a highly exergonic process, which accounts for ~ 60–90% of the total ATP production. Carbohydrates, mainly glucose, lactate, and pyruvate, contribute the remaining 10–40% of the total ATP production (Bing et al., 1954; Lopaschuk et al., 1994; van der Vusse et al., 2000; Wisneski et al., 1987).

1.1.1 Glucose fatty acid cycle

The glucose fatty acid cycle, also called the "Randle cycle", was discovered and described in the rat heart by Phillip Randle. It shows the relationship between glucose and FFA oxidation and their mutual regulation (Randle, 1998). Generally, the resting heart metabolism is highly economic, the FFA oxidation predominates and the glucose oxidation pathway is partially reduced. The reduction of glucose metabolism is performed on three levels: i) limitation of glucose uptake, ii) suppression of glycolytic enzymes activities, and iii) inhibition of glucose oxidation. The high concentration of the FFA in blood decreases the glucose uptake into cardiac cells (Nuutila et al., 1992; Randle et al., 1964), which leads to low intracellular concentration of glucose and to a decrease in hexokinase (HK) activity. The HK activity can be inhibited by its product glucose-6-phosphate (G-6-P), which is not further metabolized through glycolysis due to an inhibition of the phosphofructokinase 1 (PFK1). The

G-6-P can be further incorporated into glycogen due to a stimulation of glycogen synthase (McNulty et al., 1995; Villar-Palasi and Guinovart, 1997) or degraded *via* the pentose phosphate pathway to generate nicotinamide adenine dinucleotide phosphate (NADPH) required for the FFA biosynthesis. The PFK1 activity is limited by an increased concentration of the citrate generated from the acetyl coenzym A (acetyl-CoA) during FFA oxidation (Garland et al., 1963). The massive production of the acetyl-CoA and the nicotinamide adenine dinucleotide (NADH) during the FFA oxidation also results in the suppression of the pyruvate dehydrogenase (PDH) activity, which blocks the formation of acetyl-CoA from pyruvate, thereby inhibiting the glucose oxidation. In addition, the acetyl-CoA and the NADH indirectly activates the pyruvate dehydrogenase kinase complex (PDC), which phosphorylates and thus inactivates the PDH (Kerbey et al., 1976; Randle and Priestman, 1996).

On the other hand, increased cardiac workload, e.g., under physical exercise or hypoxia, results in the increase of glucose uptake into cells. The intracellular glucose is immediately phosphorylated by the HK to produce the G-6-P, which is directly degraded in the glycolytic pathway. The PFK1 is strongly activated by the increased intracellular levels of the adenosine 5'monophosphate (AMP), adenosine 5'diphosphate (ADP), iorganic phopshate (Pi), or fructose-2,6-bisphosphate (F-2,6-P). The F-2,6-P is a by-product of the glycolysis forming from the fructose-6-phosphate by the enzyme phosphofruktokinase 2 (PFK2) (Kantor et al., 2001; Katz, 2006). The PFK2 can be stimulated *via* phosphorylation by the 5'AMP-activated protein kinase (AMPK), whose activity increases with the rise in the AMP/ATP ratio (Marsin et al., 2000, 2002).

The fatty acid cycle is primarily controlled by the ATP production, which depends on an adequate supply of electrons for the respiratory chain generating a force for the ATP synthase. Thus, the $NADH/NAD^+$ ratio represents one of the main regulators, which is maintained by the combined action of glycolysis and tricarboxylic acid cycle (TCA cycle). The increase in the $NADH/NAD^+$ ratio leads to an increase in the ATP/ADP ratio, which under decreased energy demands results in the inhibition of enzymes in the TCA cycle and glycolysis. An increased concentration of the ATP also inhibits the AMPK and thus reduces the FFA oxidation in favor of the FFA biosynthesis occurring in liver and the triacylglycerol synthesis in the adipose tissue. Conversely, under increased energy demands, the TCA cycle

and the glycolysis are stimulated by an increased concentration of free ADP and Ca^{2+} ions (Voet and Voet, 2010).

However, the fatty acid cycle is also affected by the substrate availability, (patho)physiological interventions, and hormones. Therefore, the regulation of the substrate contribution to the total ATP production represents a far more complex mechanism, including the allosteric control, phosphorylation/dephosphorylation system, and the regulation of gene expression (discussed in more detail in Hue and Taegtmeyer, 2009).

1.1.2 Substrate utilization

The heart possesses a metabolic flexibility and ability to utilize different substrates depending on the specific conditions and the actual substrate availability. While the metabolism of the fetal heart is predominantly glycolytic, the metabolism of the adult heart is highly oxidative. However, the adult heart is able to switch its preferences to glucose utilization under low oxygen supply, such as under increased workload, hypoxia, or ischemia. Under these conditions, the glucose coming from the glycogen breakdown and from blood, due to an increased glucose uptake, is metabolized in anaerobic glycolysis. The lactate from blood also becomes a preferential fuel of the heart under these conditions (Opie, 2004) and can contribute between 5 and 10% of the total ATP production depending on workload (Allard et al., 1994). An increased intracellular level of lactate can inhibit the glucose oxidation *via* the inhibition of PDH and under the severe oxygen deprivation even glycolysis *via* the inhibition of PFK1 (Depre et al., 1993, 1998b). Beside the conditions dependent on the oxygen concentration, the high carbohydrate diet leads to a higher consumption of the glucose. The heart can also utilize amino acids and ketone bodies, mainly during the prolonged starvation and diabetic acidosis (Voet and Voet, 2010).

1.1.3 Hormonal regulation

The fatty acid cycle is also controlled by the pancreatic hormones, insulin and glucagon, together with the adrenal hormones, adrenaline and noradrenaline. The glucagon is secreted during the fasting and energy demand states, when the blood glucose concentration is

lowering under the 5 mM. The glucagon and adrenaline promotes glycogenolysis by the activating adenylate cyclase signaling pathway. An increased concentration of the 3'-5'-cyclic adenosine monophosphate (cAMP) activates the protein kinase A (PKA), which accelerates, through the phosphorylation-dephosphorylation cascade, the glycogen breakdown and inhibits the glycogen synthesis (Goodwin et al., 1995). The glucagon and adrenaline also regulate the FFA metabolism *via* the cAMP-dependent phosphorylation cascade. They stimulate expression and activity of FFA oxidation enzymes and decrease the expression and activity of enzymes involved in the lipid biosynthesis. In the adipose tissue, there is an increased hydrolysis of triacylglycerols leading to the raise of blood FFA levels, which increases the entry of the FFA into target tissues and activates the β-oxidation pathway. The energy demand states bring an increasing AMP concentration resulting in the elevation of the active AMPK, which blocks the malonyl-CoA inhibition of the fatty acyl-CoA transport into mitochondria. The malonyl-CoA is a product of the acetyl-CoA-carboxylase, which regulates the FFA oxidation in the heart (Awan and Saggerson, 1993). Adrenaline also promotes the glycogenolysis by stimulating the phospholipase C (PLC) and by increasing concentration of related second messengers, such as the inositol-1,4,5-triphosphate (IP_3), diacylglycerol (DAG), and Ca^{2+} ions. In contrast to glucagon, the insulin is secreted during the fed and resting states, when the blood concentration of glucose is increasing above 5 mM. The insulin has the opposite effect causing the stimulation of glucose transport into the cell and its utilization together with the glycogen formation. The insulin also supports the synthesis of the FFA and triacylglycerols, while reducing the glycogenolysis and the FFA oxidation (Lawson and Uyeda, 1987; Moule and Denton, 1997; Watanabe et al., 1984; Witters et al., 1988). In addition to glucagon-insulin system, the hormonal regulation of the fatty acid cycle can also include thyroid hormones, sex hormones, and growth hormone (reviewed in Clegg, 2012; McAninch and Bianco, 2014; Møller and Jørgensen, 2009; Randle, 1964).

1.2 Creatine kinase

The creatine kinase (CK) represents a major phosphagen system transporting the ATP in the heart. The impaired CK function is thus a sign of many human diseases, including cardiovascular disorders. The substantial reduction or loss of the CK activity or creatine content cause the insufficiency of energy metabolism observed in the heart failure or the myocardial infarction (Bottomley et al., 2009).

The CK catalyzes a reversible reaction: phosphocreatine (PCr^{2-}) + $MgADP^{-}$ + protons (H^{+}) ↔ $MgATP^{2-}$ + creatine (Cr) (Kenyon and Reed, 1983; Watts, 1973), thus in the case of acute energy needs the CK regenerates the ATP for muscle contraction and, on the other hand, the CK provides supply of the PCr. The PCr is a unique molecule, which possesses a higher phosphoryl transfer potential than the ATP and therefore represents a major source of phosphoryl groups for immediate ATP regeneration. Another advantage of PCr molecule is that it is much smaller than the ATP and can easily and faster diffuse between sites of its production and consumption. The PCr is also metabolically inert and thus it can accumulate in the cell to high concentrations without affecting regulatory feedback loops. The concentration of PCr in the resting muscle is ~ 25 mM, 6-fold higher than the ATP concentration (Ingwall, 2002), which covers the first ten seconds of the high performance before the ATP production by glycolysis and oxidative phosphorylation is activated.

Mammalian tissues express four CK isoforms: the cytoplasmic muscle CK (CKM), cytoplasmic brain CK (CKB), ubiquitous mitochondrial CK (mtCKu), and sarcomeric mitochondrial CK isoform (mtCKs) (reviewed in Wallimann et al., 1992, 1998). The cytosolic CK isoforms are mainly located in close proximity of ATPases, where they keep a high local ATP/ADP ratio and optimal pH for ATPase activities. The mitochondrial CK isoforms highly support mitochondrial oxidative phosphorylation by increasing the ADP availability for complex V of the respiratory chain, which can protect cells against apoptosis (Meyer et al., 2006; Santiago et al., 2008). All CK isoforms create together a highly compartmentalized phosphocreatine/creatine kinase (PCr/CK) system, which transports energy from sites of production, mitochondria, to various sites of utilization, including the sarcomere, sarcoplasmic reticulum, and plasma membrane (*Figure 1*) (Bessman and Carpenter, 1985; Rossi et al., 1990), and thus controls the energy flow within cells. Therefore, the PCr/CK system functions

as a spatial and temporal energy buffer maintaining the cellular energy homeostasis (reviewed in Saks et al., 1996; Schlattner et al., 2006; Ventura-Clapier et al., 1994; Wallimann et al., 1992).

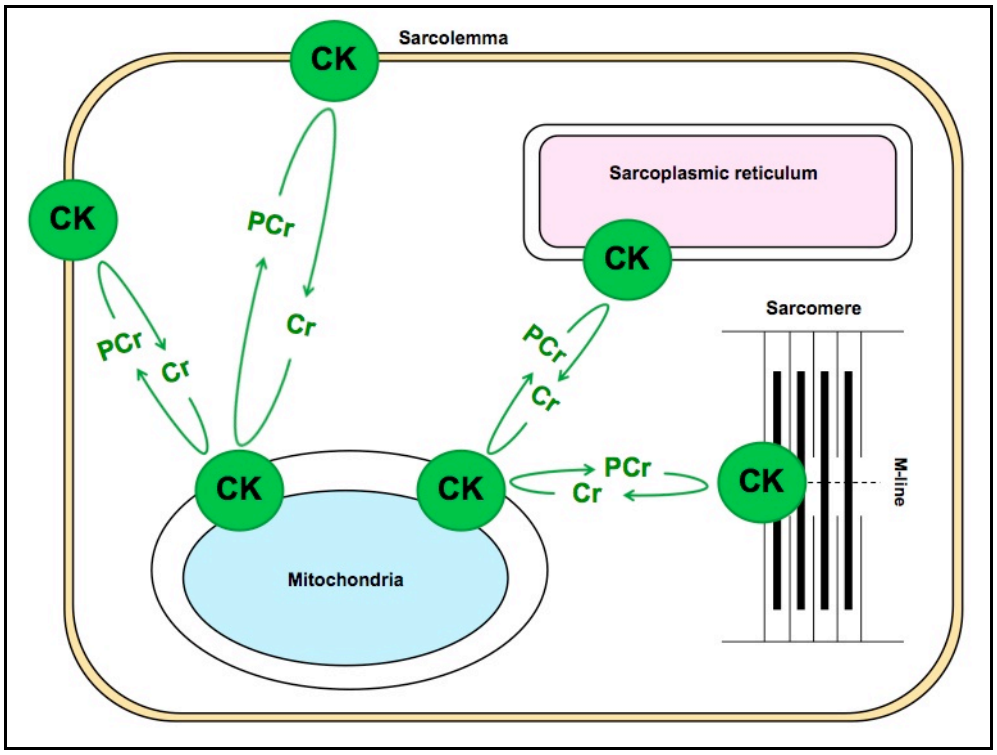

Figure 1: *The PCr/CK system transporting energy from sites of production (mitochondria) to sites of utilization (sarcomere, sarcoplasmic reticulum, and sarcolemma). The CK, creatine kinase; PCr, phosphocreatine; Cr, creatine.*

1.2.1 Cytosolic CK isoforms

The cytosolic isoforms CKM and CKB are encoded by *ckm* and *ckb* genes (Bertin et al., 2007; National Center for Biotechnology Information, NCBI), respectively. The CKM gene is located on the chromosome 19 in human tissues (NCBI #1158) (Nigro et al., 1987; Stallings et al., 1988) and on the chromosome 1 in rat tissues (NCBI #24265). The CKB gene is located on the chromosome 14 in human tissues (NCBI #1152) (Chern et al., 1980) and on the chromosome 6 in rat tissues (NCBI #24264). Both of these isoforms create homo-dimers CKMM and CKBB or the hetero-dimer CKMB. The CKM is predominantly expressed in the heart and skeletal muscles, while the CKB is abundantly expressed in the brain, heart, smooth muscle, uterus, placenta, colon (Trask et al., 1988), as well as in tumors (Gazdar et al., 1981) and most permanent cell lines (Kuzhikandathil and Molloy, 1994; Ritchie et al., 1991). In the skeletal muscle, the CKB expression is very low and an absolute absence of the CKB is observed in bone marrow (Mariman et al., 1987).

In the adult cardiac muscle, the CKM represents a major isoform, whereas the minor isoform CKB is predominantly expressed in early developmental stages of the heart. In adulthood it functions as a backup enzyme, which is over-expressed under conditions of metabolic challenge occurring during the hypertrophy (Ingwall, 1984; Smith et al., 1990), hypertension (Fontanet et al., 1991; Pauletto et al., 1989; Smith et al., 1990), and hypoxia (Letout et al., 2005; Pissarek et al., 1997; Waskova-Arnostova et al., 2014). At the cellular level, the CKB is present in the cytosol as a solubilized molecule unable to bind with intracellular structures in contrast to the CKM, which can associate with intracellular structures through its $NH_{(2)}$-terminal lysine charge-clamps (*Figure 2*) (Hornemann et al., 2000).

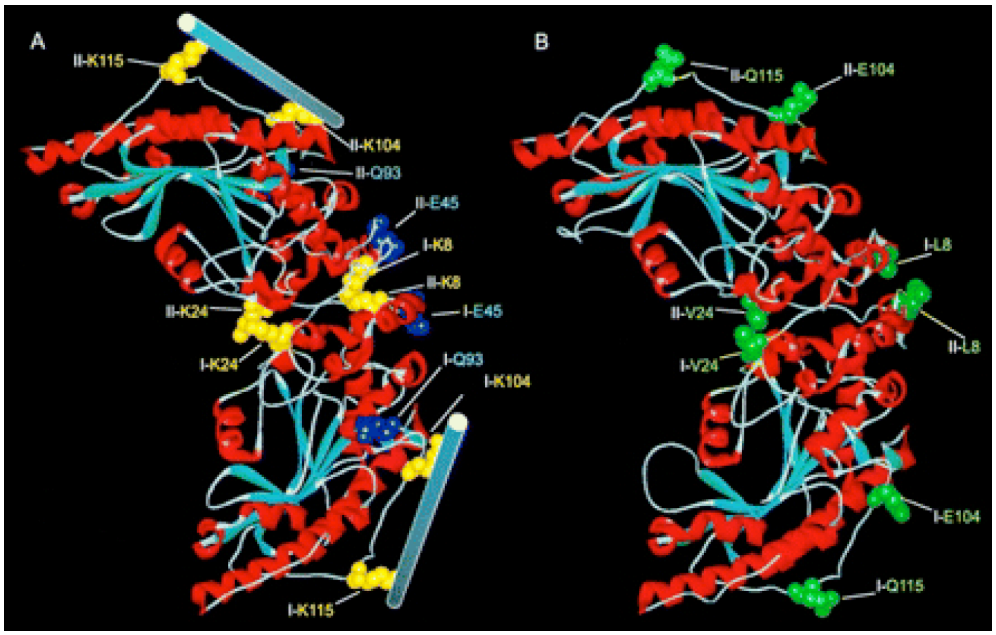

Figure 2: *The x-ray structure of the (A) rabbit CKM isoenzyme dimer (Rao et al., 1998) and the (B) chicken CKB isoenzyme dimer (Eder et al., 1999). The lysine residues K8, K24, K104, K115, which have been identified to be responsible for the isoenzyme-specific interaction of the CKM with the M-line are shown in yellow (A) and the homologous residues in the CKB isoform L8, V24, E104, and Q115 are depicted in green (B). The figure is reprinted from ©2000. Hornemann et al. Journal of Cell Biology. 149:1225-1234, with permission from Dr. Hornemann and Rockefeller University Press.*

The CKM was found to bind into the myofibrillar M-line of the sarcomere (Turner et al., 1973; Wallimann et al., 1977, 1978, 1983a; b), where it is functionally coupled to the acto-myosin ATPase (Gregor et al., 2003; Wallimann et al., 1984) and regenerates the ATP for cardiac contraction (Ventura-Clapier et al., 1987a; b). The CKM delivers preferentially ~ 40% of the ATP to this ATPase and the maximal efficiency of this process is in a slightly acidic pH (Gregor et al., 2003; Zurmanova et al., 2007). This privileged exchange of substrates and products is called a "substrate channeling", which is also observed in mitochondrial CK isoforms. The CKM is also located within I-band of the sarcomere (Wegmann et al., 1992), where it is functionally linked to the glycolysis and glycogenolysis (Scopes, 1973; Van Waarde et al., 1990). The functional coupling between the CKM and the pyruvate kinase (PK) has been reported as the exchange of the phosphate between the PCr and the phosphoenolpyruvate without a change in the ATP (Dillon and Clark, 1990). The direct association of the CKM with the PFK at low pH, which occurs for example under increased

workload, has also been described (Kraft et al., 2000). Other glycolytic enzymes, such as the aldolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), and phosphoglycerate kinase (PGK) are also able to interact with the F-actin-tropomyosin complex (Arnold and Pette, 1970; Bronstein and Knull, 1981). The potential involvement of these enzymes in the functional coupling of the CKM with glycolysis could be speculated.

The CKM have been found to be associated with the sarcoplasmic reticulum (Baskin and Deamer, 1970; Khan et al., 1972; Rossi et al., 1990; Sharov et al., 1977), where it is functionally coupled to the ATP-dependent Ca^{2+} pump (Korge et al., 1993) regulating local ATP/ADP ratios and supporting Ca^{2+} release (Rossi et al., 1990) due to the fact that IP_3 receptor is allosterically regulated by intracellular concentration of the ATP (Ferris et al., 1990). In addition, the CKM also regenerates the ATP for the Ca^{2+} ATPase (SERCA) in the sarcoplasmic reticulum, enabling a faster Ca^{2+} re-uptake and thereby causing a faster cardiac relaxation.

Some amounts of CKM have also been found at the sarcolemma membrane (Saks et al., 1977), where it is functionally coupled to the ATP-dependent Na^+/K^+ pump (Grosse et al., 1980; Saks et al., 1977) optimizing the Na^+/K^+ antiport across the membrane due to the immediate re-phosphorylation of the ADP produced in the Na^+/K^+ ATPase reaction. The CKM also physically binds to the sarcolemmal ATP-dependent K^+ (K_{ATP}) channel and inhibits its opening, i.e., K^+ flux across the membrane, *via* maintaining a high ATP level within the close proximity of the channel (Crawford et al., 2002). It has been recently described that the CKM is able to interact with the Na^+/Ca^{2+} exchanger (NCX) and regulates its activity (Yang et al., 2010). It is well known that the antiport of Na^+ and Ca^{2+} ions is not dependent on the ATP. However, it has been shown that the NCX activity is stimulated by the ATP (Collins et al., 1992), thus the CKM may be involved in the regulation of the NCX activity *via* increasing the ATP/ADP ratio around this exchanger.

Given these observations, the CKM is critically involved in the regulation of ion fluxes taking place during the excitation-contraction coupling and plays an important role in the synchronizing electrical signals on the membrane with cellular metabolic states.

1.2.2 Mitochondrial CK isoforms

Two mtCK isoforms are encoded by three genes in human and by two genes in rat tissues. While the mtCKu isoform is encoded by two genes *ckmt1A* (NCBI #548596) and *ckmt1B* (NCBI #1159), located near each other on the chromosome 15 in human tissues, only a single gene *ckmt1* on the chromosome 3 has been identified in rat tissues (NCBI #29593). The mtCKs isoform is encoded in human and rat tissues by a single gene *ckmt2* located on the chromosome 5 (NCBI #1160) and 2 (NCBI #688698), respectively (Bertin et al., 2007). The mitochondrial mtCKu is abundantly expressed in conjunction with the CKB in the intestine, brain, kidney, placenta, and during pregnancy in uterus (Friedman and Perryman, 1991; Payne et al., 1993). A very low expression level of the mtCKu was detected in aorta and in sarcomeric tissues. No expression was detected for liver and lung. The mtCKs isoform is expressed only in slow-oxidative and fast-oxidative-glycolytic skeletal muscles and in the heart, but not in any other organs.

The mtCK is located in the cristae and in the intermembrane space of mitochondria (*Figure 3*) (Beutner et al., 1998; Brdiczka et al., 1998).

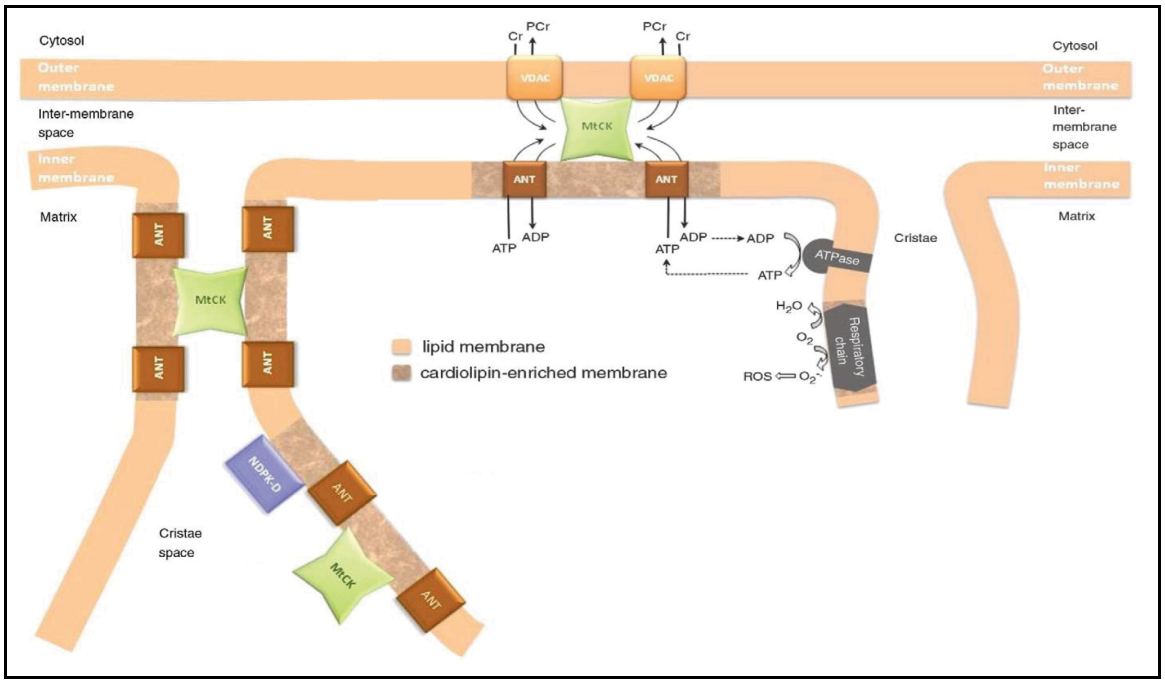

Figure 3: *The mtCK localization in the cristae and in the intermembrane space of mitochondria. The MtCK, mitochondrial CK; ANT, adenine nucleotide translocase; VDAC, voltage dependent anion channel; NDPK-D, nucleoside dihosphate kinase D. The figure is reprinted and slightly modified from the paper Mitochondrial kinases and their molecular interaction with cardiolipin, Schlattner et al., 2009, Biochim Biophys Acta. 2009 Oct;1788(10):2032-47, Figure 2 and 3. The figure is republished with kind permission from Prof. Schlattner and Elsevier B.V.*

The mtCK exists as two inter-convertible forms, a homodimer and a homooctamer (Marcillat et al., 1987). Only the octameric form of the mtCK can bind to the outer face of the inner mitochondrial membrane, dimers can no longer interact with anionic phospholipids (Rojo et al., 1991; Soboll et al., 1999). In the inner mitochondrial membrane, the mtCK binds with a high affinity to a phospholipid cardiolipin (Müller et al., 1985, 1986; Rojo et al., 1991; Schlame and Augustin, 1985; Stachowiak et al., 1996) *via* following a two-step mechanism. The first step is the electrostatically driven adsorption of positively charged lysine residues of mtCK exposed on the top and bottom faces of the octameric cube by the negatively charged cardiolipin head (Schlattner et al., 2004). The second step involves the protein insertion between lipids, which is generally associated with hydrophobic interactions (Maniti et al., 2010). The cardiolipin also interacts with other proteins binding peripherally to the inner mitochondrial membrane, including cytochrome *c* (Gonzalvez et al., 2008; Rytomaa et al., 1992; Salamon and Tollin, 1996a; b) or nucleoside diphosphate kinase (NDPK-D) (Tokarska-

Schlattner et al., 2008), and further with trans-membrane protein complexes of the redox chain and transporters like the adenine nucleotide translocase (ANT), an ATP/ADP antiporter (Schlattner et al., 2009). The cardiolipin controls retention of the cytochrome *c* in the cristae while apoptotic stimuli induce the cardiolipin oxidation and the cytochrome *c* release (Kagan et al., 2005). During apoptosis, an oxidized cardiolipin is transferred from the inner to the outer mitochondrial membrane and this transfer can be facilitated by the mtCK and NDPK-D *via* liposomal vesicle bridges (Epand et al., 2007). On the outer mitochondrial membrane, the cardiolipin provides a recognition site for Bcl-2 pro-apoptotic proteins (Schlattner et al., 2009), acts as a mitochondrial receptor for the tBID (Lutter et al., 2000), and regulates the oligomerization of pro-apoptotic BAK and BAX proteins (Kuwana et al., 2002). In fact, cardiolipin-protein interactions are important not only for activity and structural integrity of mitochondrial inner membrane proteins, but also for the subunit assembly, supercomplex formation, and apoptosis prevention (Claypool et al., 2008; Pfeiffer et al., 2003; Schlame et al., 2000; Zhang et al., 2002). The mtCK-cardiolipin interaction is very important for the "substrate channeling" and for the maintaining of mitochondrial ultrastructure and morphology (Lenz et al., 2007; Speer et al., 2005).

The mtCK is able to cross-link membranes to form contact sites, where it is functionally coupled with the ANT (Schlattner et al., 1998; Wyss et al., 1992) in the inner mitochondrial membrane and physically interacts with a voltage dependent anion channel (VDAC) in the outer mitochondrial membrane (*Figure 3*) (Brdiczka et al., 1994, 2006; Schlattner et al., 2001). Another functional property common to the mtCK is the ability to stimulate the respiration through local synthesis of the ADP in the intermembrane and cristae space *via* functional coupling with the ANT (*Figure 3*) (Dolder et al., 2001; Frey and Mannella, 2000; Saks et al., 2003, 2004; Vendelin et al., 2004). In peripheral contact sites, the co-localization of mtCK with ANT, the direct interaction of mtCK with VDAC, and possibly also the diffusion limitations at the outer mitochondrial membrane (Gellerich and Kunz, 1987) create a micro-compartment that maintains the "substrate channeling" (Dolder et al., 2003; Saks et al., 1985, 2007; Vendelin et al., 2004). However, the locally produced ADP is immediately re-imported into the mitochondrial matrix space *via* the ANT and the PCr is then released into the cytosol *via* the VDAC. The mitochondrial metabolism would not be regulated by the intracellular free ADP concentration *per se*, but rather by the intra-mitochondrial ADP

production triggered *via* the cytosolic Cr (Kay et al., 2000; Saks et al., 2004, 2007). This could indicate the presence of a factor or regulator in the outer mitochondrial membrane, which helps to restrict free ADP diffusion from the intermembrane space (Veksler et al., 1995). In cristae, the mtCK associates only with the ANT and it would still allow the "substrate channeling" between these two proteins. The Cr and PCr molecules, however, have to diffuse along the cristae space to reach the VDAC (Schlattner et al., 2009). The ATP/ADP recycling is restricted mostly to mitochondrial intermembrane and matrix spaces, while the Cr and PCr are recycled between mitochondrial intermembrane and cytoplasmic spaces (Saks et al., 2010). Many experimental evidences point to the role of the tubulin in regulating the VDAC (Appaix et al., 2003; Carre et al., 2002). Recently, functional interaction of the tubulin with the VDAC was revealed by applying biophysical and oxygraphic methods by Rostovtseva and colleagues (Rostovtseva and Bezrukov, 2008; Rostovtseva et al., 2008) and by Monge and his team (Monge et al., 2008). Rostovtseva and her colleagues proposed the model for the tubulin-VDAC interaction in which the negatively charged C-terminal tail of the tubulin penetrates into the channel lumen due to the interaction with a positively charged domain of the VDAC (Rostovtseva et al., 2008). Guzun et al. (2009) showed that tubulin and other cytoskeletal proteins like desmin (Capetanaki, 2002; Linden et al., 2001) and plectin (Reipert et al., 1999) selectively limit the VDAC permeability, restricting mostly the ATP and ADP but not the Cr or PCr (Guzun et al., 2009). The strongly decreased permeability of the outer mitochondrial membrane for adenine nucleotides significantly enhances the functional coupling between the mtCK and ANT increasing the rate of the ADP and ATP recycling in the mitochondrial matrix-intermembrane space. Especially interesting and important is the significantly enhanced apparent affinity of the mtCK for the Cr in cells *in situ* (Guzun et al., 2009). It appeared that all ATP produced by the oxidative phosphorylation is practically completely used for the PCr production and the ADP is rapidly channeled back through the ANT to the mitochondrial matrix (Timohhina et al., 2009). The selective regulation of diffusion barrier functions is highly important for the structural and functional organization of the energy metabolism.

The mtCK-ANT-VDAC complex highly supports the oxidative phosphorylation of mitochondria by the PCr pool restoration in the cytosol and by increasing the availability of ADP for complex V of the respiratory chain. The complex also decreases the membrane

potential and hyper-production of reactive oxygen species (ROS) (Jacobus and Lehninger, 1973; Jacobus and Saks, 1982; Kernec et al., 1996; O'Gorman et al., 1996; Saks et al., 1991; Wyss et al., 1992), and it diminishes the opening of the mitochondrial permeability transition (MPT) pore, a well known trigger of apoptosis (Dolder et al., 2001, 2003; Kowaltowski et al., 2001; Meyer et al., 2006; Saks et al., 2007). Recently, biochemical and structural studies by Peter Pederson's laboratory (Chen et al., 2004; Ko et al., 2003) resulted in the discovery of the "ATP synthasome", a complex consisting of the ATP synthase, phosphate carrier (PIC), and ANT (Ko et al., 2003). From a physiological point of view, the "ATP synthasome" (*Figure 4*) allows a continuous production of the ATP by transporting Pi and ADP into the matrix directly to the active sites of the ATP synthase and an immediate release of the ATP into the intermembrane space (Pedersen, 2007b).

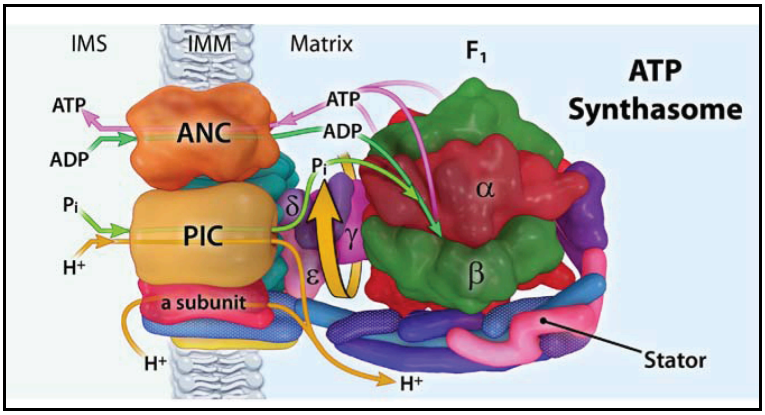

Figure 4: *The ATP synthasome. The ANC, adenine nucleotide carrier; PIC, phosphate carrier; IMS, intermembrane space; IMM, innermitochondrial membrane. The figure is reprinted from the paper Transport ATPases into the year 2008: a brief overview related to types, structures, functions and roles in health and disease, Pedersen PL, Springer and J Bioenerg Biomembr, 2007 Dec;39(5-6):349-55, Figure 2a. The figure is republished with kind permission from Prof. Pedersen and Springer Science and Business Media.*

Therefore, there seems to be the supercomplex of ATP synthasome-mtCK-VDAC-tubulin localized in contact sites of cardiac mitochondria, which regulates the respiration. This whole complex was shortly named "mitochondrial interactosome" (*Figure 5*) (Timohhina et al., 2009).

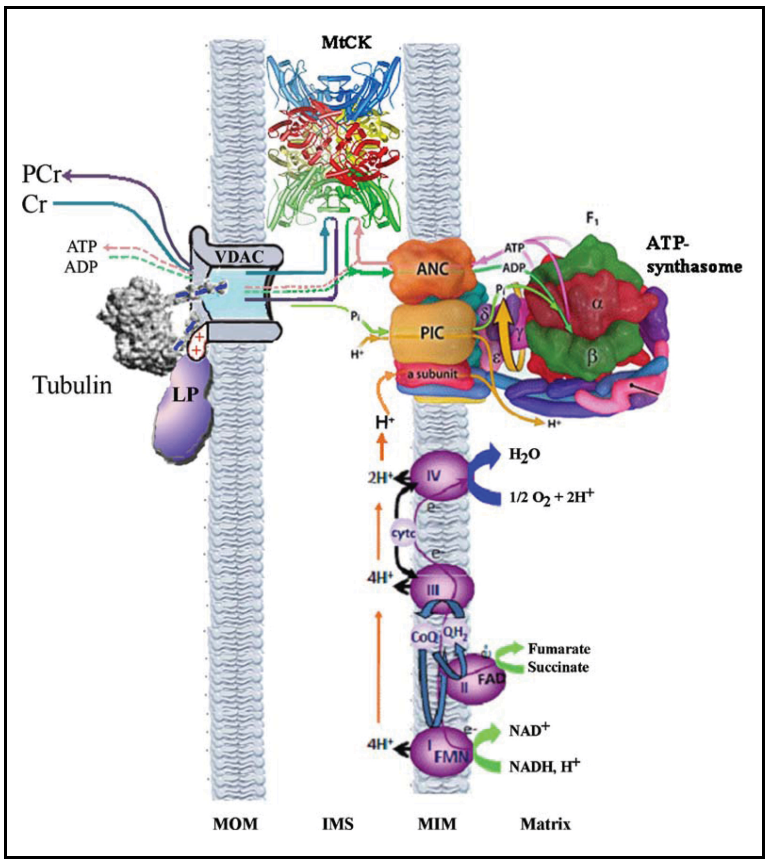

Figure 5: *The mitochondrial interactosome. The MtCK, mitochondrial CK; VDAC, voltage dependent anion channel; ANC, adenine nucleotide carrier; PIC, phosphate carrier; PCr, phosphocreatine; Cr, creatine; MOM, mitochondrial outer membrane; IMS, intermembrane space; MIM, mitochondrial inner membrane; LP, linker protein. The figure is reprinted from the paper Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome, Timohhina et al., Springer and J Bioenerg Biomembr. 2009 Jun;41(3):259-75, Figure 9. The figure is republished with kind permission from Springer Science and Business Media.*

This mitochondrial interactosome may in some cases include also supercomplexes of the respiratory chain (Lenaz and Genova, 2007; Vonck and Schäfer, 2009). Along the cristae membranes the mitochondrial interactosome contains only the mtCK and the "ATP synthasome" (Timohhina et al., 2009). The mitochondrial interactosome regulates the interaction between mitochondrial cycles of adenine nucleotides and PCr/Cr cycles in the cytoplasm of the heart, skeletal muscles and brain cells. Changes in the mitochondrial interactosome may lead to severe pathology (Saks et al., 2010).

1.2.3 Regulation of CK expression and activity

The expression of the CK is regulated at the transcriptional, post-transcriptional, translational, and the post-translational level. The cytosolic CKs and the mitochondrial CKs are often expressed co-ordinately: the mtCKs together with the CKM and the mtCKu with the CKB. The best-studied area is the development of skeletal and cardiac muscles. The most of the myogenic factors are involved in the expression of all CK isoforms. It is generally known, that during myogenesis and cardiogenesis, the cytosolic CK isoforms undergo a transition from the major fetal CKBB isoforms *via* an intermediate CKMB heterodimer to the major adult CKMM isoform (Adamson, 1976; Dym and Yaffe, 1979; Ingwall, 1976; Trask and Billadello, 1990). Transcription factors responsible for this process in skeletal and cardiac muscles come mainly from MyoD and MEF families of proteins, respectively (reviewed in Qin et al., 1998). The CKM gene expression is particularly controlled by transcription factors, such as the MyoD1 (Davis et al., 1987), myogenin (Edmondson and Olson, 1989; Wright et al., 1989), Myf5 (Braun et al., 1989), and MRF4/herculin/Myf6 (Braun et al., 1990; Miner and Wold, 1990; Rhodes and Konieczny, 1989), and by transcription enhancers MEF1, MEF2, and MADS box (Buskin et al., 1985; Molkentin et al., 1995). The CKB contains the same common TA-rich recognition protein-binding regulatory elements as the CKM (Horlick et al., 1990), thus the CKB expression can be controlled by the same transcription MEF enhancers that are expressed in the brain (Lin et al., 1996; Lyons et al., 1995). However, it has been observed that the MyoD1 and myogenin by themselves did not result in a significant increase in the CKM expression during skeletal myogenesis, suggesting that the presence of other transcription factors and enhancers is required (Amacher et al., 1993; Lyons et al., 1991). These may include the homeodomain transcription factor Six4, which was found as a trans-activator of the CKM enhancer (Himeda et al., 2004). Another important transcriptional regulator is the p53, which is activated during the myoblast differentiation and together with the MyoD stimulates the CKM gene expression in muscle cells (Jackson et al., 1998; Tamir and Bengal, 1998) and represses transcription of the CKB (Zhao et al., 1994). These findings may suggest the possible explanation for the switch between cytosolic CK isoforms during development stages. Another developmental factor described as a negative regulator of the CKB gene expression is the PARP-1 (Chen et al., 2010). The CKB up-regulation is related to

an impaired energy supply occurring during pathophysiological states and diseases (Fontanet et al., 1991; Ingwall, 1984; Letout et al., 2005; Neubauer et al., 1998; Pauletto et al., 1989; Pissarek et al., 1997; Smith et al., 1990), when the CKM is mostly inhibited. It has been shown that an ischemia (Mehta et al., 1988) or a mechanical overload (Tsika et al., 1995) lead to a significant decrease in the CKM expression. The CKM activity was also lower in the failing human myocardium compared to the physiological status (Nascimben et al., 1996). In the study of Boheler and Dillmann (1988), two *in vitro* translation products of the CKM, CKMa and CKMb, representing the more acidic and basic forms, respectively, have been found, but only the CKMb isoform decreased with cardiac hypertrophy. The isoelectric micro-heterogenity for the CKM has been also described in other studies both *in vitro* and *in vivo* (Rosenberg et al., 1982; Schweinfest et al., 1982), suggesting that the transcriptional or translational control for the CKM is much far more complicated than previously believed and that multiple mRNAs could arise from the alternative splicing (Boheler and Dillmann, 1988) under stress conditions. The β-adrenergic stimulation by the isoproterenol induced a switch in the CK gene expression from the CKM to the CKB, which is characteristic for the hypertrophied or failing heart. This may be interpreted as an adaptive mechanism making energy transduction *via* the CK more efficient at times of increased metabolic demand (Hammerschmidt et al., 2000). These findings could be explained by the stimulation of the signaling pathway *via* the cAMP and PKA to activate transcription of the CKB (Korge et al., 1993; Korge and Campbell, 1994; Kuzhikandathil and Molloy, 1994; Minajeva et al., 1996; Rossi et al., 1990). Willis et al. (2005) have first reported a role for the AP2 factor, which is activated by the cAMP, in the regulation of the CKB transcription (Willis et al., 2005). The transcription factor Sp1, acting through the estrogen signaling pathway, was also found to regulate the CKB gene expression (Wu-Peng et al., 1992). Moreover, Ch'ng et al. (1990) have suggested that translation of the CKB is regulated by the binding of a soluble factor or factors to the 3' UTR (Ch'ng et al., 1990). The post-translational regulation of the CKB is controlled by the protein kinase C (PKC)-mediated phosphorylation, which increases the CKB activity (Chida et al., 1990; Hemmer et al., 1993). Nevertheless, the PKC also modulates an activity of the CKM, but in this case the phosphorylation decreases the CKM activity (Lin et al., 2009). Beside the PKC, the AMPK can also phosphorylate the CKM and thus reduce its activity (Ponticos et al., 1998). Other post-translational modifications, such as the autophosphorylation

and the nucleotidylation of CK molecules, have been also identified as effective modulators of its catalytic function (David and Haley, 1999; Hemmer et al., 1995; Stolz et al., 2002). The possible physiological explanation for the up-regulation of the CKB under the stress conditions is that this isoform possesses unique characteristics relevant to the maintenance of ATP concentrations in specialized instances of high energy demand (Mahadevan et al., 1984). The phosphorylated CKB has an increased affinity for the PCr (Quest et al., 1990). This higher affinity may increase the CKB ability to transfer the high-energy phosphate from the PCr to the ADP, thereby providing a higher ATP supply for ATP-requiring processes at low PCr levels (Hemmer et al., 1993). The CK isoforms are highly susceptible to an oxidative stress and to a free radical damage (Konorev et al., 1998; Koufen et al., 1999; Mekhfi et al., 1996; Wendt et al., 2003). It has been reported that the CKM activity was depressed after the incubation of myofibrils with ROS (Mekhfi et al., 1996). These results suggest that ROS mainly alters the myofibril-bound CKM probably by the oxidation of -SH bonds between cysteine residues in each CK monomer, which may be followed by the CKM dissociation from the M-line. The interaction of the myofibrillar CKM in the M-line is strongly pH dependent (Zurmanova et al., 2007), which is reflected in the "substrate channeling" between the CKM and the myosin ATPase (Gregor et al., 2003). Such CKM inactivation results in a decrease in the intra-myofibrillar ATP/ADP ratio (Mekhfi et al., 1996), leading to a higher concentration of the ADP and Pi, which causes an inhibition of ATPases. In addition, Zhao et al. (2007) have observed that the CKM exists in two forms, in the reduced form (R-CK) and in the oxidized form (O-CK). In contrast to the R-CK, the O-CK contains an intra-chain disulfide bond in each subunit and it has decreased catalytic activity. Surprisingly, the O-CK, unlike the R-CK, cannot interact with the M-line protein myomesin and can be rapidly ubiquitinylated. The O-CK has been also shown to be a negative regulator of the R-CK (Zhao et al., 2007). Likewise the CKM, the mtCK can be ubiquitinylated (Kwon et al., 2010) and it is also very sensitive to free radicals. Previous studies have described that free radicals of the X-ray-induced water radiolysis and peroxynitrite cause the dissociation of mtCK octamers into dimers, which are unable to bind into the mitochondrial membrane (Koufen et al., 1999; Wendt et al., 2003). The dissociation of the mtCK from mitochondria results in an impairment of the mitochondrial architecture and the oxidative phosphorylation (Soboll et al., 1999). A comparison of the mtCKu with the mtCKs revealed that the mtCKu is much stable and less

susceptible to the peroxynitrite than the mtCKs (Wendt et al., 2003). The study of Kaasik et al. (1999) showed that a nitric oxide (NO) is able to inhibit cardiac energy production *via* inhibition of the mtCK (Kaasik et al., 1999). On the other hand, it has been reported that expression and activity of the mtCKs increased after a chronic stress, such as hypoxia (Waskova-Arnostova et al., 2014). The activation of mtCKs indicated that the energy supply was damaged after a chronic stress and the complementary mechanism had to operate to protect cardiomyocytes from a stress-induced injury (Liu et al., 2004). The CK catalytic activity or the reversibility of the CK reaction can be further regulated by the availability of its cofactors, such as Mg^{2+} and Mn^{2+} (Noda, 1958), or inhibited by –SH group reagents (Font et al., 1983), -$SO4^{2-}$ (Cain and Davies, 1962), as well as by iron ions concentration (Korge and Campbell, 1993).

1.2.4 Intracellular phosphotransfer network

The PCr/CK system represents a major phosphotransfer system contributing to 89% of the total ATP turnover rate in the heart (Dzeja et al., 1996). The adenylate kinase (AK) phosphagen system also facilitates the transfer of high-energy phosphoryls and the signal communication between mitochondria and sites of utilization. The AK catalyzes the reversible reaction: 2 ADP ↔ ATP + AMP and contributes to 10% of the total ATP turnover rate (Dzeja et al., 1996; Zeleznikar et al., 1990). Under the conditions, when the CK is inactive or absent, the AK can partially substitute its function to maintain the intracellular phosphotransfer. It has been reported an increased contribution of the AK-catalyzed phosphotransfer to the total ATP turnover in the failing heart, when the contribution by the CK dropped to 40%. However, the compensation provided by the AK was only partial, the CK and the AK together contributed only 60% to 65% of the total ATP turnover in failing hearts (Dzeja et al., 1999). The rest 35-40% of the ATP turnover may be mediated by other phosphotransfer enzymes. Beside the phosphotransfer role, the AK also facilitates communication of mitochondrial signals to the sarcolemmal K_{ATP} channel under the stress conditions, such as the hypoxia or ischemia (Carrasco et al., 2001; Deutsch et al., 1991). The AK promotes opening of the K_{ATP} channel by hydrolyzing the ATP and producing the ADP around the channel environment and thus increases the flux of K^+ ions from cells (Carrasco et al., 2001), which is associated with their

electrical instability (O'Rourke et al., 1994; Wilde and Janse, 1994). Studies on genetically manipulated animals lacking the gene for one of the CK isoforms or for both major CK isoforms have revealed increased phosphotransfer enzymes enabled to compensate the CK deficiency.

The CKM absence, which eliminates ~ 71% of the total CK activity (Dzeja et al., 2011), does not cause any changes in cardiac energy metabolism or left ventricular contractile function, even during an increased cardiac workload (Saupe et al., 1998). The CKM function is compensated by an increased phosphotransfer through the CKB isoform and glycolytic enzymes, such as the HK. The AK phosphotransfer flux remains unchanged in this case (Dzeja et al., 2011). It has been also described that mitochondria of CKM knockout mice had a higher sensitivity to the cellular ADP and thus a higher permeability of the outer mitochondrial membrane, indicating a greater participation of the mtCKs in cytosolic processes (Veksler et al., 1995) and its higher phosphotransfer activity (Dzeja et al., 2011). However, it should be also mentioned that Saupe et al. (2000) have demonstrated no compensatory increase in the CKB isoform in hearts lacking the CKM (Saupe et al., 2000). Comparing the heart and skeletal muscles, which possess nearly undetectable amount of the CKB and conversely nearly 100% of the CKM, the CKM deficiency is mainly compensated by an increased contribution of the cytosolic AK1 and also glycolytic phosphotransfers, such as the GAPDH, PK, and PGK, to the total cellular ATP turnover (Dzeja et al., 2004; Ventura-Clapier et al., 1995). Studies on fast-twich skeletal muscles of mice deficient in the CKM have also shown an increase in the mitochondrial volume (Kaasik et al., 2003; van Deursen et al., 1993) and respiration rate (Veksler et al., 1995) to accelerate delivery and production of the ATP required for the muscle contraction. However, this adaptation cannot fully compensate the lack of the CKM and as a result, it leads to a weak communication between the ATP synthase and acto-myosin ATPase associated with a lower energetic efficiency.

The combined loss of the CKM and the mtCKs is in the heart still well compensated due to the activation of other phosphotransfer systems. Double CK-knockout is associated with a significant decrease, by over 88%, of the total CK-phosphoryl capability, which eliminates ~ 96% of the total CK activity. The remaining CKB represents only 12% of this phosphoryl capability. Kassik et al. (2001) suggested that the combined knockout of the CKM and mtCKs results in a direct channeling between mitochondria and ATP-utilizing structures

(Kaasik et al., 2001). These authors have later observed an increased content of the intermyofibrillar mitochondria, an increased oxidative capacity and aerobic metabolism, which supports their previous findings (Kaasik et al., 2003). It is generally known that the heart has a much higher content of mitochondria, which is needed for a greater capacity of the aerobic ATP synthesis. Nevertheless, the recent work of Dzeja et al. (2011) showed that the combined loss of the CKM and mtCKs led to the involvement of more phosphotranfer systems. They have observed an increased phosphotransfer flux through the CKB and the glycolytic phosphotransfer system, primarily *via* the HK, further an increased phosphotransfer flux through the AK system, and also elevated guanosine 5'triphosphate (GTP) turnover through the nucleoside monophosphate kinase (NMPK) and NDPK (Dzeja et al., 2011). Like the CK, glycolytic enzymes are also involved in the regulation of the K_{ATP} channels (Dzeja and Terzic, 1998; Weiss and Lamp, 1987) and they are able to support the SERCA activity (Boehm et al., 2000; Xu et al., 1995). Beside the maintaining of the high-phosphoryl group transfer, the enzyme carbonic anhydrase maintains the H^+ and pH homeostasis and reduces H^+ concentration around ATPases, protecting them against inhibition (Dodgson et al., 1980; Geers and Gros, 1991; Stewart et al., 1999). This way, the carbonic anhydrase substitutes the CK role in CKM/mtCKs-deficient hearts. These results demonstrate the existence of comprehensive compensation mechanisms keeping the energy homeostasis in hearts with partial CK deficiency. However, the communication between ATP-consuming and ATP-generating cellular sites is compromised, being reflected in the increased cardiac workload. The heart lacking both major CK isoforms, the CKM and mtCKs, has a reduced ability to respond to β-adrenergic stimulation (Crozatier et al., 2002) and is more vulnerable to ischemia-reperfusion (I/R) injury (Spindler et al., 2004).

The deletion of the mtCKs alone resulted in similar changes in the high-energy phosphate metabolism as observed in CKM/mtCKs knockout animals, suggesting that the mtCKs is an enzyme primarily responsible for the energy homeostasis in the heart (Spindler et al., 2002). This is in contrary with results from mtCKs-deficient skeletal muscles, where the deletion of the mtCKs has no significant effect on the deterioration or change in the PCr/CK system and the ATP turnover. This suggests that the mtCKs role in the stimulation of oxidative phosphorylation is well compensated by an alternate process including the mitochondrial NDPK-D, AK2 or AK3, and glycolytic enzymes (Dzeja et al., 2004). The

opposite findings between cardiac and skeletal muscles could be related to the different proportion of two major CK isoforms to the total CK activity. The CKM and the mtCKs constitute 61% and 25% of the total CK activity in the heart, respectively, while 98% and 2% in skeletal muscles, respectively (Bittl et al., 1987). Therefore, the heart and skeletal muscles differ significantly in response to the absence of any of the major CK isoforms. The lack of both CKM and mtCKs in skeletal muscles causes also progressive changes in the energy homeostasis (Momken et al., 2005; Steeghs et al., 1997, 1998), even in the decrease of PCr turnover and the Pi compartmentalization. Hereby, the skeletal muscles become more sensitive to metabolic stress, like the heart (Dzeja et al., 2004). These observations indicate the importance of the PCr/CK system as a central phosphotransfer system in excitable tissues.

Taken together, the intracellular phosphotransfer network, including the PCr/CK system, AK, glycolytic enzymes, guanine nucleotide system, and carbonic anhydrase, represents a functional cellular bioenergetics infrastructure supporting an efficient high-energy phosphoryl transfer, ionic homeostasis, and metabolic signal communication (*Figure 6*) (reviewed in Dzeja and Terzic, 2003). The disruption of this network leads to many serious cardiovascular diseases (Bottomley et al., 2009; Dzeja et al., 1999; Ingwall et al., 1985; Ingwall, 2009; Janssen et al., 2000; Pucar et al., 2002).

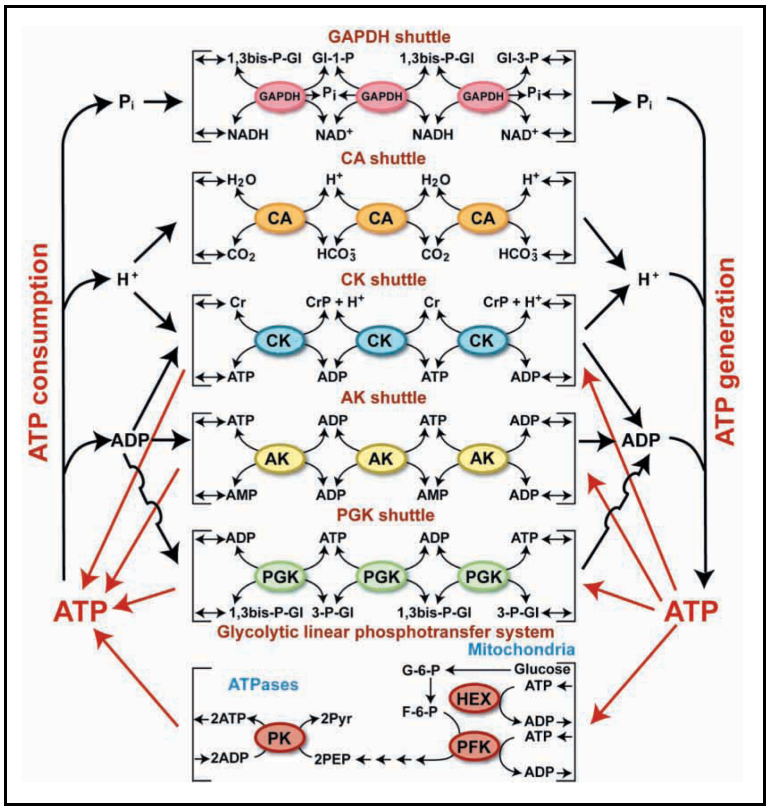

Figure 6: *The intracellular phosphotransfer network. The GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 1,3 bis-P-Gl, 1,3 bis-phosphoglycerate; Gl-3-P, glyceraldehyde-3-phosphate; CA, carbonic anhydrase; CK, creatine kinase; Cr, creatine; CrP, phosphocreatine; AK, adenylate kinase; PGK, phosphoglycerate kinase; 3-P-Gl, 3-phosphoglycerate; PK, pyruvate kinase; HEX, hexokinase; G-6-P, glucose-6-phosphate; PFK, phosphofructokinase; F-6-P, fructose-6-phosphate; PEP, phosphoenol pyruvate; PYR, pyruvate. The figure is republished with permission of Prof. Dzeja and The FASEB Journal from the paper Phosphotransfer networks and cellular energetics, Dzeja and Terzic, J Exp Biol. 2003 Jun;206(Pt 12):2039-47, Figure 3; permission conveyed through Copyright Clearance Center, Inc.*

1.3 Hexokinase

The Hexokinase is a key glycolytic enzyme, which keeps the intracellular glucose concentration at a low level by phosphorylating glucose to G-6-P, thereby regulating glucose fluxes into cardiomyocytes. The HK is thus essential for the regulation of the glucose utilization and for the maintenance of the glucose uptake (Wilson, 2003). Depending on the current conditions, the produced G-6-P is further metabolized *via* triosa-phosphate pathway, which is the primary glycolytic pathway for the ATP generation. Alternatively, it can be processed through the pentosa-phosphate pathway to generate NADPH and precursors for biosynthetic reactions or it may be used for a glycogen synthesis (Voet and Voet, 2010). As mentioned previously, the HK is an integral part of the intracellular phosphotransfer network, where it tightly co-operates with the CK and in some cases can compensates the CK function. Beside its metabolic function, the HK also plays an important role in anti-apoptotic processes. The HK bound to mitochondria also stimulates oxidative phosphorylation and thus prevents ROS over-production and oxidative stress similarly as the mtCKs. In addition, the HK competes with pro-apoptotic proteins for particular binding site on the mitochondria, thereby inhibiting apoptosis. Dysfunction of the HK results in decreased cardiac function and heart is then more susceptible to the I/R injury (Wu et al., 2011). The dissociation of HK2 from mitochondria is associated with an extensive decrease in the tolerance of the heart to the I/R insult (Smeele et al., 2011). The reduced HK2 also impairs the function of skeletal muscles (Smeele et al., 2010, 2012). On the other hand, the over-expression of the HK2 protects the cancer cells against cell death (Ahmad et al., 2002; Azoulay-Zohar et al., 2004; Sun et al., 2008). Therefore this enzyme could be a potential candidate for therapeutic interventions in cardiovascular as well as in cancer research. The experimental intervention directed at increasing and/or maintaining of the HK bond with mitochondria appears to be a promising cardioprotective approach.

1.3.1 Glucose transport

The glucose represents one of the important energy substrates in cardiac and skeletal muscles. The glucose is transported into cardiomyocytes by facilitative glucose transporters (GLUTs), which are developmentally and hormonally regulated (reviewed in Abel, 2004). The predominant GLUTs expressed in the heart are GLUT1 and GLUT4 isoforms. The GLUT1 is the most abundant isoform in the embryonic heart, whereas the GLUT4 predominates in the adult heart (Smoak and Branch, 2000; Studelska et al., 1992). Regulation of the GLUT1 transcription is mediated by similar transcription factors involved in the CKB transcription regulation. The GLUT4 transcription is controlled by transcription MEF enhancers, as the CKM gene expression, and mainly by thyroid hormones. After the birth the circulation of thyroid hormones significantly increases (Lompre et al., 1984), which results in increased GLUT4 expression. The GLUT1 and GLUT4 expressions are also regulated by the insulin, however, the GLUT4 is the major regulator of insulin stimulated glucose uptake in the heart (Kraegen et al., 1993). During fasting, when the insulin is low, cardiac FFA utilization increases and glucose utilization decreases, leading to repression of both GLUT1 and GLUT4 expressions. However, the isoforms can differ in their response to pathophysiological conditions. Up-regulation of GLUT1 is associated with hypertrophy (Tian et al., 2001), hypoxia (Sivitz et al., 1992), or ischemia (Brosius et al., 1997). The regulation of the GLUT1 expression during hypoxia and ischemia could probably be mediated by the AMPK (Tian et al., 2001) or by hypoxia inducible factor, HIF1 (Chen et al., 2001a). Generally, hypoxia activates the expression of fetal genes including also the myosin heavy chain (MyHC) β and the CKB (Letout et al., 2005; Pissarek et al., 1997; Waskova-Arnostova et al., 2014). The GLUT4 expression is elevated by endurance exercise (Ren et al., 1994) and conversely is down-regulated by hypertrophy (Paternostro et al., 1995). The effect of hypoxia or ischemia on the GLUT4 expression probably depends on the duration and intensity of the stimulus. The more pronounced changes have been observed in the translocation of GLUTs into the plasma membrane. Under the resting conditions, the GLUT1 and GLUT4 are located in the intracellular vesicles and are recruited into plasma membranes under an increased level of catecholamines and adrenergic activation (Egert et al., 1999a; Rattigan et al., 1991), under ischemia, hypoxia, and increased concentration of insulin (Egert et al., 1999b; Sun et al.,

1994). Beside GLUT1 and GLUT4 isoforms, other members of the glucose transporter family have been determined in the heart. The GLUT3 in the fetal heart (Grover-McKay et al., 1999) and the GLUT12, which is insulin-responsive (Macheda et al., 2002; Rogers et al., 2002), GLUT11 (Doege et al., 2001; Wu et al., 2002b), GLUT8 (Doege et al., 2000), and GLUT10 (Dawson et al., 2001) in the adult heart.

1.3.2 Hexokinase isoforms

The mammalian tissues express four isoforms of hexokinase: HK1, HK2, HK3, and HK4 (Gonzalez et al., 1964; Katzen and Schimke, 1965), which differ in their kinetic and regulatory properties, transcriptional regulation, tissues distribution, and subcellular location. HK1, HK2, and HK3 are 100 kDa molecules, while HK4 is only 50 kDa molecule. It has been speculated that 100 kDa HKs arose by duplication and fusion from an ancestral 50 kDa HK, while HK4 probably separated before this process and therefore remains smaller than other mammalian HK isoforms (Cardenas et al., 1998). Each of the three 100 kDa HKs consist of two parts: C-terminal half, which is catalytically active, and N-terminal half of whose function differs between isoforms. While HK1 and HK2 possess the 15 hydrophobic amino acid residues on their N-terminal half enabling them to interact with mitochondria (Fiek et al., 1982; Kurokawa et al., 1982; Linden et al., 1982; Rose and Warms, 1967), HK3 lacks this sequence and is mainly soluble. The HK3 has been also found in a perinuclear compartment (Preller and Wilson, 1992; Wilson, 2003), but its specific metabolic role remains unclear. In addition, HK2 contains two active sites, one on the C-terminal half, as HK1 and HK3, and the second one on the N-terminal half (Ardehali et al., 1996; Printz et al., 1997; Tsai and Wilson, 1996), which again points to a distinct function. The structural features are naturally related to kinetic parameters of the enzyme. All 100 kDa HK isoforms are inhibited by their product G-6-P. Only HK1 inhibition can be antagonized by a low Pi concentration. The regulatory effect of the Pi is based on the direct competitive inhibitory mechanism attenuating the G-6-P binding to the N-terminal half of the enzyme at physiological concentrations (Ellison et al., 1974, 1975; Rose and Warms, 1967; Tsai and Wilson, 1995) and an indirect displacement of the G-6-P from the catalytic site on the C-terminal half (Fang et al., 1998). The HK2 is more sensitive to the inhibition by its G-6-P product, because it is not antagonized by the Pi at the

N-terminal domain (Lueck and Fromm, 1974). The HK3 is also inhibited by the Pi at all concentrations (Wilson, 2003) and moreover, the HK3 may be also inhibited by a high concentration of glucose (above 0.2 mM), but the physiological significance has not been completely resolved (Radojkovic and Ureta, 1987). On the other hand, the HK4 cannot be physiologically inhibited by its product G-6-P. The HK4 has the highest *Km* for glucose from all four HKs, thus it requires a high concentration of glucose for the reaction, which is related to a high value of *Vmax* indicating a low concentration of G-6-P (Niemeyer et al., 1975; Storer and Cornish-Bowden, 1977). The HK4 also phosphorylates glucose faster than other three HKs, which can be due to a different interaction of ATP molecule within a binding site of the enzyme (Kumar et al., 2012). The structural, kinetic, and regulatory differences are associated with tissue distribution. The HK4 is mainly expressed in liver and pancreatic tissue, where it acts as a glucose sensor. However, some studies have reported no HK4 activity in liver of several species (Ureta et al., 1973, 1975), even mammals (Ureta et al., 1981). The lack of the HK4 in liver could represent the adaptive phenotype based on the diet that is high in protein and low in carbohydrate content and thus these species, particularly carnivores, have mild glucose intolerance and reduced insulin sensitivity (discussed in more detail in Schermerhorn, 2013). The HK4 has also different promoters in liver and pancreas, allowing tissue-specific gene regulation (Andreone et al., 1989; Magnuson and Shelton, 1989). The HK3 has the highest level in lung, kidney, and liver, in other tissues is absent or present in very low concentrations (Ureta, 1982). The HK1 is expressed in most tissues, but predominantly in brain and muscles. The increased energy demand activates glycolysis, thereby increasing the Pi/G-6-P ratio, which prevents the inhibition of HK1, i.e., its activity increases. Moreover, the low *Km* for glucose allows to retain more than 70% of full HK1 activity at glucose concentration as low as 0.1 mM (Niemeyer et al., 1975; Storer and Cornish-Bowden, 1977). This is important especially for brain, which is almost entirely dependent on glucose metabolism (Clarke and Sokoloff, 1999). The HK2 predominates in insulin-sensitive tissues, such as skeletal muscles, heart, and adipose tissue. This isoform is also expressed at high levels in many tumors (Shinohara et al., 1991) that exhibits a “Warburg effect”, metabolizes glucose to lactic acid at a high rate even in the presence of oxygen (Warburg, 1956).

The heart expresses two HK isoforms, HK1 and HK2 (Aubert-Foucher et al., 1984; Burcelin et al., 1993). HK1 and HK2 isoforms are encoded by *hk1* and *hk2* genes,

respectively. The HK1 gene is located on the chromosome 10 in human tissues (NCBI #3098) and on the chromosome 20 in rat tissues (NCBI #25058). The HK2 gene is located on the chromosome 2 in human tissues (NCBI #3099) and on the chromosome 4 in rat tissues (NCBI #25059). The HK1 and HK2 share a high degree of functional similarity but there are some differences. As already mentioned, they differ in their substrate affinity and subcellular localization reflecting their different metabolic roles (John et al., 2011; Mathupala et al., 2009; White and Wilson, 1990). It has been suggested that under the physiological conditions, the HK1 is predominantly bound to the outer mitochondrial membrane and channels glucose towards glycolysis (Wilson, 1997, 2003), whereas the HK2 is mainly soluble and controls glycogen formation (John et al., 2011; Sebastian et al., 2000; Wilson, 2003). Distribution of the HK2 between the cytosol and the mitochondria is dynamically regulated by the glucose availability (John et al., 2011). Increasing glucose uptake *via* the main glucose transporter GLUT4 (Abel, 2004; Postic et al., 1994) mediates the interaction of the HK2 with the outer mitochondrial membrane (*Figure 7*) (Southworth et al., 2007). This interaction enhances the affinity of HK2 to the ATP (Aubert-Foucher et al., 1984; Bustamante and Pedersen, 1977; Depre et al., 1998b; Wilson, 1997) and makes the HK2 less sensitive to inhibition by its product G-6-P, which is further metabolized in the glycolytic pathway (Bustamante and Pedersen, 1977; Bustamante et al., 1981; Depre et al., 1998b).

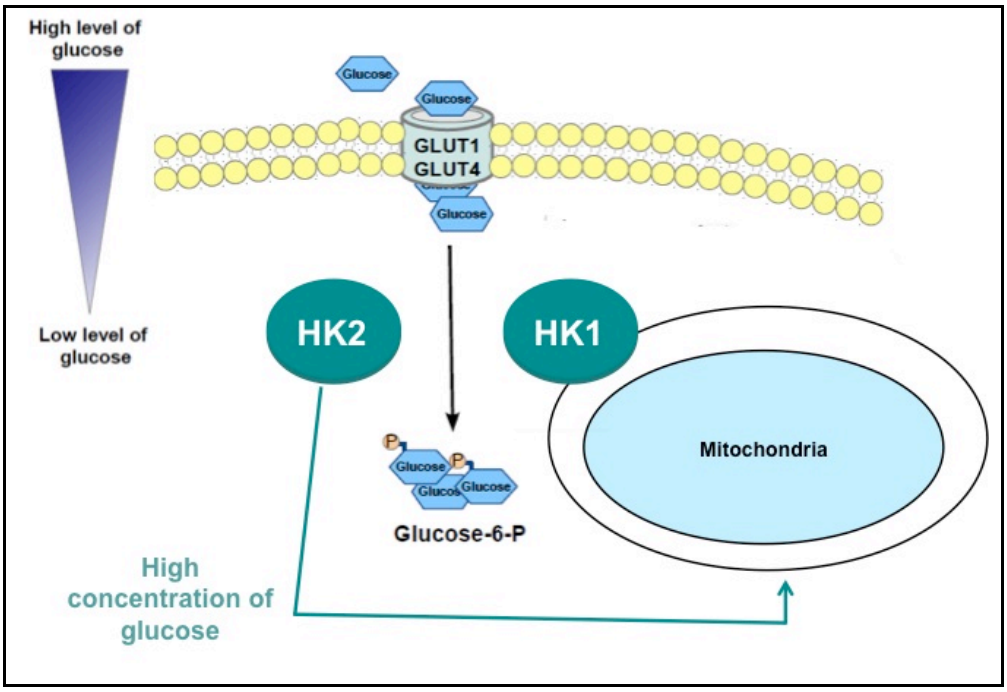

Figure 7: *The intracellular distribution of HK1 and HK2 isoforms. The HK1, hexokinase1; HK2, hexokinase 2; GLUT, glucose transporter; Glucose-6-P, glucose-6-phosphate.*

Both HK isoforms bind to mitochondria *via* VDAC (Anflous-Pharayra et al., 2007; Rosano, 2011; Shoshan-Barmatz et al., 2009), where highly support oxidative phosphorylation by increasing the availability of ADP for complex V of the respiratory chain (Chen et al., 2001b). This helps to maintain a suitably low membrane potential and prevent the over-production of ROS (da-Silva et al., 2004; Santiago et al., 2008). Recently, the existence of a complex VDAC-HK2-ATP synthasome (Ko et al., 2003) in cancer cells has been shown allowing the HK2 a preferential access to mitochondrially generated ATP, thus enhancing oxidative phosphorylation as well as glycolysis (*Figure 8*) (Chen et al., 2004; Pedersen, 2007b; a, 2008).

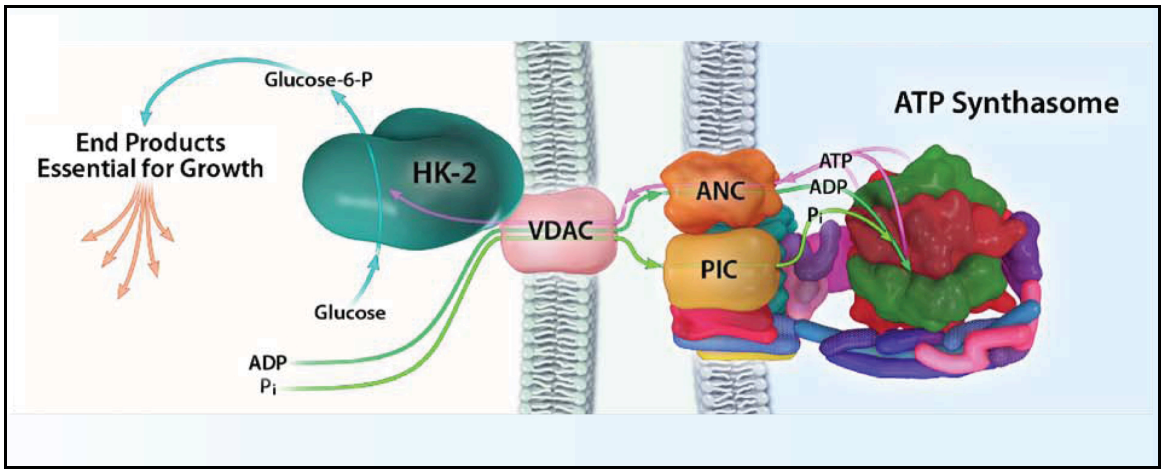

Figure 8: *The complex of the VDAC, HK2, and ATP synthasome. The HK2, hexokinase 2; VDAC, voltage dependent anion channel; ANC, adenine nucleotide carrier; PIC, phosphate carrier; MOM, mitochondrial outer membrane; IMS, intermembrane space; MIM, mitochondrial inner membrane. The figure is reprinted from the paper Warburg, me and Hexokinase 2: Multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg Effect", i.e., elevated glycolysis in the presence of oxygen, Pedersen PL, Springer and J Bioenerg Biomembr. 2007 Jun;39(3):211-22, Figure 1g. The figure is republished with kind permission from Prof. Pedersen and Springer Science and Business Media.*

The HK bound to mitochondria also reduces the probability of apoptosis initiation (Azoulay-Zohar et al., 2004; Majewski et al., 2004a; Pastorino and Hoek, 2003) by inhibiting the binding of the pro-apoptotic protein BAX to the outer mitochondrial membrane (Pastorino et al., 2002), thereby preventing MPT pore opening and cytochrome *c* release (Azoulay-Zohar et al., 2004; Beutner et al., 1998; Miyamoto et al., 2008). It has been shown that the HK1 can also inhibit the formation of active pro-apoptotic caspases and block the mitochondrial step of tumor necrosis factor (TNF)-mediated cell death (Schindler and Foley, 2010). These observations suggest that, besides its critical involvement in the regulation of glucose metabolism, the HK could play a crucial role in protective signaling pathways. It has also been shown that the over-expression of full-length HK results in protection against cell death (Ahmad et al., 2002; Azoulay-Zohar et al., 2004; Sun et al., 2008). Both HK isoforms are essential for the normal physiological function of the heart and are not mutually substitutable. The depletion of the HK1 isoform results in caspase-8-dependent cell death in response to the TNF (Schindler and Foley, 2010), which indicates a strong pro-survival function of this enzyme. The physiological importance of the HK1 probably lies to a high degree in the association of this isoform with mitochondria, because its function in glycolysis could be

replaced by HK2. Accordingly, the binding of the HK2 to mitochondria has been shown to be necessary for the normal function of mitochondria. A recent study demonstrated that the displacement of the HK2 from isolated mitochondria using an artificial peptide resulted in the enhanced release of cytochrome *c* upon treatment with the recombinant tBID, a membrane-targeted death ligand (Shulga et al., 2009). In addition, the disruption of the HK2 binding to mitochondria blocks ischemic preconditioning and may cause myocardial necrosis (Smeele et al., 2011) or apoptosis (Chiara et al., 2008).

1.3.3 Regulation of HK expression and activity

The regulation of the HK expression is different between tissues and depends on the availability of substrates (Griffin et al., 1991). The HK1 and HK2 also differ in their subcellular locations and affinities for their substrates (John et al., 2011; Mathupala et al., 2009; White and Wilson, 1990), which can be reflected by their distinct and varying responses to actual conditions. In Addition, promoter regions of the HK1 and HK2 are distinct in character and responsive to different transcription factors (Heikkinen et al., 2000; Liu and Wilson, 1997; Mathupala et al., 1995; Osawa et al., 1996a; b; White et al., 1996). The regulatory mechanism of the HK1 gene expression is not completely understood. It has been demonstrated that Sp sites may have the functional significance as cis-regulatory elements in this case (Liu and Wilson, 1997; White et al., 1996). The Sp factors are also involved in the regulation of the GLUT1 transcription (Santalucia et al., 1999). This could indicate a relationship in the regulation of gene expression between the HK1 and GLUT1. There could therefore be a similar relationship between the HK2 and GLUT4. Studies focused on the HK2 promoter from normal and cancer cells have revealed that the hypoxic conditions, glucose, insulin, glucagon, catecholamines, cAMP, and p53 activate the HK2 transcription (Mathupala et al., 1995, 1997, 2001; Rempel et al., 1996; Osawa et al., 1995, 1996a). From these transcription factors, only the insulin and hypoxia also increase the GLUT4 transcription (Chou et al., 2004; Olson and Pessin, 1995), while the cAMP decreases the GLUT4 gene expression (Flores-Riveros et al., 1993). A common transcription factor for the HK2 and GLUT4 could be the AMPK, which is activated during endurance training (Stephens et al., 2002) as well as hypoxia (Emerling et al., 2009; Jing et al., 2008; Mungai et al., 2011), under

the same conditions as the expression of the HK2 (Koval et al., 1998; Waskova-Arnostova et al., 2014) and the GLUT4 (Chou et al., 2004; Ren et al., 1994). Moreover, a chronic activation of the AMPK by the adenosine analog, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), led to an increased expression of the HK2 (Stoppani et al., 2002) as well as GLUT4 (Holmes et al., 1999). In addition to expression, the AMPK also increases the HK activity (Holmes et al., 1999), which demonstrates a more complex role of this kinase. The main regulator of the HK2 is the HIF1 (Riddle et al., 2000), which together with other co-activators, such as Myc, activates the HK2 transcription (Kenneth and Rocha, 2008). The HIF1 also positively regulates the transcription of the HK1, GLUT1, GLUT4, and other glycolytic enzymes (reviewed in (Marin-Hernandez et al., 2009; Semenza et al., 1994). Recent study has described the translocation of the HK2 into nucleus in cancer cells (Neary and Pastorino, 2010), which has so far been observed only in yeast (Randez-Gil et al., 1998). Experiments on *Saccharomyces cerevisiae* revealed that the HK2 can activate its own transcription and repress transcription of the HK1 (Rodriguez et al., 2001), suggesting its role as a transcription factor. However, this regulatory mechanism has not yet been described in mammalian cells. It has been shown that other multifunctional kinase, the AKT kinase, also regulates the expression of HK2 at the transcriptional level *via* the phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT kinase (PI3K/AKT) signaling pathway (Osawa et al., 1996b) stimulated by the insulin (Printz et al., 1993).

However, the more important role of the AKT is related to the post-translational regulation of the HK. Recently, it has been reported that the AKT kinase phosphorylates the HK2 at the Thr473 residue and stimulates its translocation into the mitochondria (Miyamoto et al., 2008). The interaction of the HK2 with the outer mitochondrial membrane enhances its binding affinity for the ATP (Bustamante and Pedersen, 1980) and the HK2 thus becomes less sensitive to inhibition by the G-6-P (Bustamante and Pedersen, 1977; Bustamante et al., 1981) and it gains preferential access to mitochondrial generated ATP (Arora and Pedersen, 1988). The effect of the AKT requires the presence of glucose (Majewski et al., 2004b). Therefore, both glucose and AKT signaling promote the binding of the HK2 to the mitochondria, thereby favoring glucose catabolism over glycogen synthesis (John et al., 2011). The association of the HK with mitochondria is enhanced under ischemic conditions (Southworth et al., 2007), by an increased level of insulin (Southworth et al., 2007; Zuurbier et al., 2005), after morphine

administration (Zuurbier et al., 2005), or under increased concentration of glucose (John et al., 2011). Nevertheless, association of the HK with mitochondria under hypoxic conditions has not yet been elucidated. The subcellular distribution of the HK2 can be also regulated by intracellular stimuli, such as pH changes. The shift of pH toward basic values increases the interaction of the HK2 with mitochondria, conversely, the acidic pH decreases this interaction (Miccoli et al., 1996). Another regulator is lactate, whose effect on the HK localization is tissue specific. In skeletal muscles, lactate can promote dissociation of the HK from mitochondria and thus also indirectly inhibits its activity, whereas in the heart, lactate does not affect neither the HK distribution, nor activity (Leite et al., 2011). This could be explained by the ability of the heart to metabolize lactate in contrast to fast-twich skeletal muscles (Voet and Voet, 2010). The same authors have also reported the inhibition of the HK activity and alteration in the distribution mediated by lactate in liver tissue, which is the main tissue for lactate degradation in Cori cycle, but independent on the glucose concentration used in the study (Leite et al., 2011). These observations suggest that lactate can also act as a positive modulator of gluconeogenesis and represent thus the universal molecule.

The HK activity differs among species and exhibits the transmural gradient in the heart (De Tata et al., 1986, 1988). The HK exhibits the maximal enzyme activity in the heart during the fetal period and only moderate changes occur during postnatal life (Andres et al., 1984; Bass et al., 2001). Changes in the total HK activity correlate with specific changes in the activity of the HK2 rather than with the activity of the HK1 (Riddle et al., 2000). The higher HK activity is associated with the binding of the HK to the mitochondria. It has been demonstrated that the HK activity doubled during the first hour after the HK translocation into the mitochondria and continued to rise thereafter (Parra et al., 1997). Mechanisms involved in the enhancement of the enzyme activity induced by the HK interaction with the mitochondria include conformational changes of the HK molecule and the Pi competition with the G-6-P at its binding site. The HK2 forms dimers in the cytosol, while the interaction of the HK2 with mitochondria requires the HK in a tetrameric form, as well as the HK1-mitochondria interaction (Mulichak et al., 1998; Wilson, 1995). Dimers or tetramers may differ in their activity (Hoggett and Kellett, 1992). Post-translational modifications, such as sumoylation (Aslanukov et al., 2006) and ubiquitinylation (Magnani et al., 1994), may affect the enzyme activity of the HK and cause its degradation. The majority of soluble HK1 in brain tissue has

been found to be ubiquitinylated at the N-terminal portion of the molecule, which may increase its susceptibility to degradation (Magnani et al., 1994) and affect its enzyme activity (Pastorino and Hoek, 2003). The HK monomer-dimer-tetramer transition and their interactions with mitochondria can further affect the conformational state of the monomers and thus change their substrate-binding affinity resulting in the increased activity of the whole complex.

The HK activity can be also affected by hormones, such as insulin (Pilkis, 1970), thyroid hormones (Kubista et al., 1971; Rosa et al., 1992), growth hormones (Bernstein and Kipnis, 1973a), and estrogen (Kostanyan and Nazaryan, 1992; Moorthy et al., 2004), further by exercise (Koval et al., 1998; O'Doherty et al., 1996), hypoxia (Daneshrad et al., 2000; Rumsey et al., 1999; Waskova-Arnostova et al., 2014), and also by age (Bernstein and Kipnis, 1973b; Ding et al., 2013). Interestingly, most studies have showed substantial changes in the HK2 isoform instead of the HK1 isoform, which emphasizes the importance of the HK2 isoform in the glycolytic metabolism control.

1.3.4 AKT kinase and glucose metabolism

The AKT kinase is involved in variety cellular processes such as cellular metabolism, cell survival, cell growth and proliferation, angiogenesis, cell migration and invasion, and it also cross-talks with other signaling pathways (reviewed in Hanada et al., 2004; Manning and Cantley, 2007; Sussman et al., 2011). The activation of the AKT kinase is a multi-step process that may be promoted by a number of stimuli. The most studied activation has been the PI3-kinase dependent activation of the AKT (Burgering and Coffer, 1995; Franke et al., 1995) shown in *Figure 9* (Shen et al., 2013).

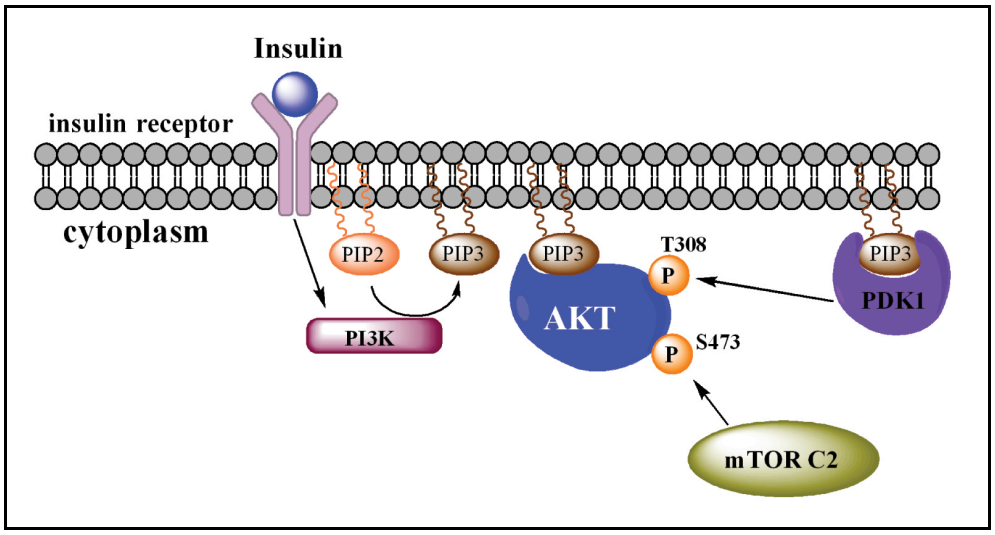

Figure 9: *The activation of the AKT kinase. The PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP2, phosphatidylinositol (4,5) bisphosphate; PIP3, phosphatidylinositol (3,4,5) trisphosphate; PDK1, phosphoinositide-dependent kinase1; mTOR C2, mammalian target of rapamycin complex 2. The figure is reprinted from the paper Dual fluorescent molecular substrates selectively report the activation, sustainability and reversibility of cellular PKB/Akt activity, Shen et al. 2013, Sci Rep. 2013;3:1697, Figure 4. The figure is republished with kind permission from Prof. Achilefu and Nature Publishing Group. This paper is licensed under a Creative Commons Attribution-Noncommercial 2.5 International License.*

In the insulin-sensitive tissues, such as cardiac and skeletal muscles, liver, and adipose tissue, the PI3-kinase dependent activation of the AKT1 stimulates metabolic pathways including the translocation of the GLUT4 to the plasma membrane facilitating glucose uptake. The PI3-kinase produces the phosphatidylinositol (3,4,5) trisphosphate (PIP_3), which interacts with the pleckstrin homology (PH) domain of the AKT1 mediating a translocation of the AKT1 to the plasma membrane. This translocation enables the phosphoinositide-dependent kinase1 (PDK1) to phosphorylate the AKT1 at the Thr308 residue in the activation loop (Alessi et al., 1996) and the mammalian target of rapamycin complex 2 (mTORC2) to phosphorylate the AKT1 at the Ser473 residue in the hydrophobic motif (Sarbassov et al., 2005). These two phosphorylated sites are necessary and sufficient for full activation of the AKT. Phosphorylation either Thr308 or Ser473 leads to a partial activation of the AKT1 *in vitro* and phosphorylation of both residues results in a synergistic activation of the enzyme (Alessi et al., 1996). Alessi and his colleagues have also demonstrated that phosphorylation of Thr308 and Ser473 phosphorylation are independent of each other in 293 cells (Alessi et al.,

1996). The Ser473 may be also phosphorylated by the mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2) (Alessi et al., 1996), but MAPKAP-K2 activation is not induced by stimuli which activate the AKT, such as the insulin-like growth factor 1 (IGF1). The MAPKAP-K2 activation is also not dependent on the PI3-kinase activation, suggesting that this kinase may not be a major kinase for the AKT Ser473 phosphorylation (Shaw et al., 1998). The Ser473 phosphorylation can dictate the differential substrate utilization (Alessi et al., 1996; Guertin et al., 2006; Jacinto et al., 2006). However, so far any specific factors or kinases that result in the Thr308 phosphorylation in the absence of the Ser473 phosphorylation have not yet been discovered (Manning and Cantley, 2007). One of the most important physiological functions of the AKT is to acutely stimulate glucose uptake in response to insulin. The AKT2, the primary isoform in insulin-responsive tissues, has been found to associate with the GLUT4 containing vesicles upon insulin stimulation of adipocytes (Calera et al., 1998), and the AKT activation leads to the GLUT4 translocation to the plasma membrane (Kohn et al., 1996). The current model is that the AKT-mediated phosphorylation of some combination of the sites on the AS160 inhibits its GAP activity. This allows a Rab-family GTPase to become GTP loaded to stimulate the GLUT4 vesicle translocation. However, recent studies suggested the AS160-independent mechanisms of regulation of this process (Bai et al., 2007), and other AKT substrates involved in various steps of the GLUT4 translocation have been identified, including the FYVE zinc finger domain kinase binding phosphatidylinositol 3-phosphate (PIKfyve kinase) (Berwick et al., 2004). The activated phospho-AKT further phosphorylates the HK2 and thus stimulates the HK2 translocation into the outer mitochondrial membrane, where the HK2 inhibits the binding of BAX protein and opening of the MPT pore. Another glycolytic enzyme phosphorylated and activated by the AKT is the cardiac-specific isoform of PFK2. The AKT phosphorylates the PFK2 on Ser466, which results in the promotion of glycolysis (Deprez et al., 1997). Another major substrate for AKT is the glycogen synthase kinase 3 (GSK3), which phosphorylates and inactivates glycogen synthase in response to insulin stimulation (Burgering and Coffer, 1995). The AKT kinase can also regulate the intracellular level of cyclic nucleotides in response to insulin. It phosphorylates the phosphodiesterase 3B (PDE3B) on Ser273, which leads to decrease of the cAMP (Kitamura et al., 1999). The anti-apoptotic effects of the AKT are related to the BAD phosphorylation on Ser136 resulting in the BAD dissociation from the Bcl-2 and Bcl-x

proteins and in the inhibition of the BAD apoptotic activity (Downward, 1999). The AKT signaling also leads to an increased production of the HIF1α and HIF2α transcription factors, at least in part, through the mTORC1-dependent translation (reviewed in Gordan and Simon, 2007; Semenza, 2003). Recently, it has been reported that both HIF1α and HIF2α are also dependent on the mTORC2 and in addition, the HIF2α gene expression is specifically dependent on the AKT2 signaling (Toschi et al., 2008).

The heart contains three AKT isoforms, AKT1, AKT2, and AKT3, but the most abundant are the AKT1 and AKT2 (Matsui and Rosenzweig, 2005). The AKT1 is mainly present in the cytosol, the AKT2 and AKT3 are localized in the mitochondrial membrane and the nucleus, respectively (Santi and Lee, 2010). The distinct subcellular localization of AKT isoforms is associated with different metabolic roles and could suggest different modes of activation and different downstream targets (discussed in more detail in Gonzalez and McGraw, 2009). The AKT1 regulates the cell proliferation and cardiac growth (Chang et al., 2010; DeBosch et al., 2006b) and plays a critical role in a cell survival (Chen et al., 2001b). The AKT2 plays a central role in the maintenance of glucose homeostasis (Cho et al., 2001) and also possesses an anti-apoptotic effect (DeBosch et al., 2006a). The AKT3 has been proposed to play a role in development and function of cardiovascular system and brain (Tschopp et al., 2005; Yang et al., 2005).

1.4 Myocardial ischemia-reperfusion injury

Cardiovascular diseases (CVDs) are one of the most common disorders of modern civilization and are leading causes of death in the world. According to the World Health Organization (WHO), the cardiovascular events cause 30% of all global deaths, representing over 17.3 million deaths per year and the number still increases (Mendis et al., 2011). It is generally accepted, that men are at greater risk of CVDs than women, but the risk for women increases after menopause (World Heart Federation, WHF). One of the proposed explanations for the gender difference is a distinct level of estrogen, which is predominant among women and may have protective effects on improving cardiovascular functions (Jousilahti et al., 1999). The risk of CVDs also increases with the age. Aging is associated with physiological and morphological changes that alter the mechanical and structural properties of the cardiac

muscle and circulatory system (Jani and Rajkumar, 2006; Minaker, 2011; Schwartz and Zipes, 2011), and thus lead to subsequently increased risk of CVDs, even in healthy asymptomatic individuals (Dantas et al., 2012). However, an increasing number of young people can develops heart problems, because of the unhealthy lifestyles, which often begins in childhood and the childhood obesity is now on the rise. There is an evidence that behavioral and metabolic factors such as tobacco smoking, physical inactivity, unhealthy diets (Finks et al., 2012; Howard and Wylie-Rosett, 2002), and the harmful use of alcohol (Yusuf et al., 2004) are one of the main risk factors causing the majority of CVDs. The long-term exposure to these risk factors results in hypertension, diabetes, dyslipidemia, and obesity, which can subsequently lead to a development of other serious diseases including renal disease, chronic respiratory disease, or cancer (Mendis et al., 2011).

The main diseases that fall under the umbrella of CVDs include diseases of the heart such as arrhythmia, cardiomyopathy, or ischemic heart disease, as well as vascular diseases of the brain, which include, for example, cerebrovascular disease and ischemic stroke, and finally diseases of blood vessels, such as hypertensive heart disease (Mendis et al., 2011). The ischemic heart disease and its' acute form, myocardial infarction, is the major cause of death and disability from all CVDs worldwide. They are responsible for nearly half of deaths caused by CVDs. The ischemic heart disease comprises almost three quarters of all cardiovascular deaths, its frequency of world mortality is ~ 13% per year, therefore it has been classified as the most serious cardiovascular disorder at this time (WHF). The ischemic heart disease is characterized by a reduced blood supply to the heart due to an atherosclerotic plaque builds up inside the coronary arteries, which can lead to the myocardial infarction. The subsequent restoration of the coronary flow, which is crucial for the viability of the myocardium, causes other complications such as an impaired heart contractility and reperfusion arrhythmias. This phenomenon is therefore termed the myocardial ischemia-reperfusion, I/R, injury and represents a common pathophysiological feature (*Figure 10*) (reviewed in Frank et al., 2012; Hausenloy and Yellon, 2013).

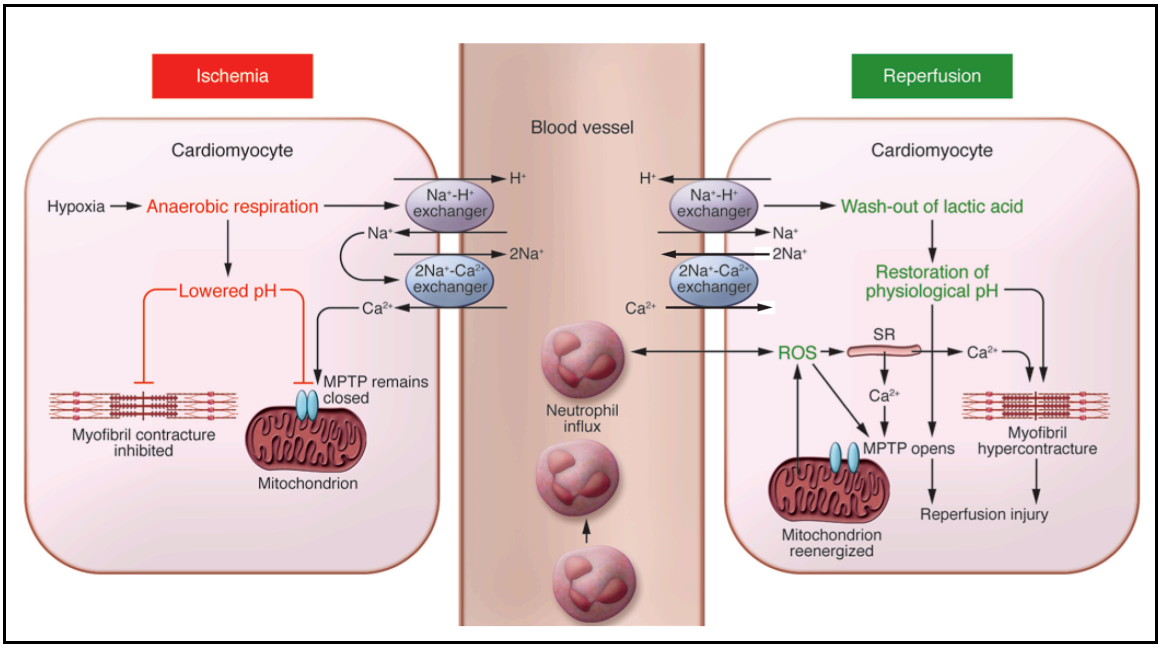

Figure 10: *The scheme of the I/R injury. The MPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; SR, sarcoplasmic reticulum. The figure is reprinted and slightly modified from the paper of Hausenloy and Yellon 2013 with permission from prof. Yellon and The Journal of Clinical Investigation.*

Myocardial ischemia-reperfusion injury: a neglected therapeutic target

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1.4.1 Myocardial ischemic injury

The ischemia occurs when the coronary artery lumen diameter is reduced by ~ 50%. Necrosis of the myocardium follows when a complete loss of blood flow occurs for more than 20 min (Kloner et al., 1983). During the prolonged ischemia, ATP levels decrease by 65% at 15 min and by 90% at 40 min (Reimer and Jennings, 1986). The absence of oxygen switches cell metabolism to anaerobic glycolysis leading to an accumulation of lactate and H^+. In contrast to the hypoxia, during the ischemia the effective removal of lactate and H^+ is significantly decreased, which results in a marked reduction of the intracellular pH and subsequently in the inhibition of glycolysis (Neely and Grotyohann, 1984). The prolonged ischemia also causes a decrease of glucose uptake (Bricknell et al., 1981) probably mediated by the cyclic guanosine 5'monophosphate (cGMP) (Depre et al., 1998a), which increases in

the ischemic heart (Depre and Hue, 1994) because of an activation of NO synthase (NOS) (Depre et al., 1997). Once the glycolysis is inhibited, glucose uptake progressively decreases, while H^+, Na^+, and Ca^+ continue to accumulate (Hausenloy and Yellon, 2013). The intracellular accumulation of H^+ activates the sarcolemmal Na^+/H^+ ion exchanger, which extrudes H^+ from the cell in exchange for Na^+ entry. The lack of the ATP during the ischemia ceases the function of the sarcolemmal Na^+/K^+ ATPase, thereby exacerbating the intracellular Na^+ overload. In response, the reverse activation of the sarcolemmal Na^+/Ca^{2+} ion exchanger results in the intracellular Ca^{2+} overloading as the cell tries to extrude Na^+ (Avkiran and Marber, 2002). The acidic conditions during the ischemia prevent the opening of the MPT pore and cardiomyocyte hypercontracture at this time (Hausenloy and Yellon, 2013). In conclusion, the ischemia inhibits oxidative phosphorylation, which leads to the depolarization of the mitochondrial membrane, ATP depletion, and inhibition of myocardial contractile function, which may further cause an acute myocardial infarction.

1.4.2 Myocardial reperfusion injury

During the reperfusion the physiological pH is rapidly restored by the wash out of lactate and H^+ due to the activation of the Na^+/H^+ exchanger as well as the Na^+/HCO^- symporter (Lemasters et al., 1996). The resultant increase in the intracellular Na^+ in turn activates the sarcolemmal $2Na^+/Ca^{2+}$ exchanger, resulting in the exchange of the intracellular Na^+ with the extracellular Ca^{2+}. A high rate of the $2Na^+/Ca^{2+}$ exchange can finally lead to the Ca^{2+} overload (Schäfer et al., 2001; Yellon and Hausenloy, 2007). The restoration pH leads to an activation of myocardial contractile function, but to a loss of the inhibitory effect on the MPT pore opening. The presence of oxygen activates the mitochondrial oxidative phosphorylation, which causes the excessive production of ROS (Ide et al., 1999, 2000; Simpson and Lucchesi, 1987). The ROS mediate dysfunction of sarcoplasmic reticulum resulting in Ca^{2+} release, which contributes to the intracellular Ca^{2+} overload (Harman and Maxwell, 1995). The ROS also causes a damage of membranes by lipid peroxidation, enzyme denaturation, and damage of DNA, which results in the ionic homeostasis instability, enzyme dysfunction, and impaired DNA replication and transcription (Ceconi et al., 1991; Galang et al., 2000). The intracellular Ca^{2+} overload causes a hypercontracture of cardiomyocytes

(Ladilov et al., 1995; Siegmund et al., 1997) and together with ROS stimulate the MPT pore opening and cytochrome *c* release leading to a cell death (Halestrap et al., 2004). The restoration of mitochondrial membrane potential drives Ca^{2+} into the mitochondria *via* the mitochondrial Ca^{2+} uniporter and subsequently induces the opening of the MPT pore. The ROS activate also an inflammatory response that leads to a further damage to viable tissue around infarct (reviewed in Marchant et al., 2012). The ROS promote the release of the pro-inflammatory factors, such as the TNFα (Cain et al., 1999; Kleinbongard et al., 2011), the nuclear factor kappa B (NFκB) (Gordon et al., 2011; Van der Heiden et al., 2010), toll-like receptors (Boyd et al., 2006), and danger–associated molecular patterns (Arslan et al., 2011). The presence of these chemoattractants results in the invasion of neutrophils (Jordan et al., 1999; Williams, 1996), which produce more ROS by the NADPH oxidase activity (Babior et al., 2002). Other sources of the ROS include the xanthine oxidase (Brown et al., 1988; Lee et al., 2009) and eNOS (Vasquez-Vivar et al., 1998; Xia et al., 1998) in endothelial cells and the cytochrome p450 (Granville et al., 2004; Sato et al., 2011). The cytochrome p450 is activated by the arachidonic acid, a product of the phospholipase A2 activity, and generates other inflammatory molecules (Granville and Gottlieb, 2006; Levick et al., 2007). Additionally, the ROS also lead to inflammasome activation in cardiac fibroblasts, which results in further inflammatory cytokine production, such as interleukin 1 β (Kawaguchi et al., 2011).

1.4.3 Cardioprotective interventions

Developing new strategies to reduce the I/R injury is currently one of the main goals of the research in cardioprotection. In this context, the myocardial ischemic pre-conditioning and post-conditioning have recently been proposed as interesting cardioprotective approaches (reviewed in Bousselmi et al., 2014). The ischemic pre-conditioning has been first documented as a cardioprotective phenomenon by Murry and his colleagues (Murry et al., 1986). The ischemic pre-conditioning is triggered by brief episodes of ischemia and reperfusion performed before the ischemic insult. Although it has been described to have a strong *in vivo* protective effect against the I/R injury (Kloner et al., 1998), its clinic application is limited. The cardioprotection persists only for hours or days (Kuzuya et al., 1993). Another disadvantage is direct stress to the heart and mechanical trauma to major vascular structures.

Another method, called the remote ischemic pre-conditioning, protects the heart indirectly *via* conditioning of other organs (Tapuria et al., 2008). Even though classical pre-conditioning may work in a clinical setting such as the heart surgery, it is not feasible in patients with acute myocardial infarction because the coronary artery is already occluded at the time of hospital admission of the patient. Zhao et al. (2003) was the first who described a phenomenon called the post-conditioning in a canine model (Zhao et al., 2003). The post-conditioning, unlike to the pre-conditioning, is induced by brief episodes of ischemia and reperfusion applied just after the ischemic insult, which allows a direct clinic application. It has been shown that the cardioprotection induced by the post-conditioning is as potent as that provided by the pre-conditioning (Darling et al., 2007; Jin et al., 2007; Staat et al., 2005; Thibault et al., 2008; Xue et al., 2010).

Beside ischemic conditionings, the adaptation to chronic hypoxia represents a potential therapeutic intervention activating similar cardioprotective mechanisms as the ischemic pre-conditioning (Neckar et al., 2002a). In addition, the myocardial infarct-size limiting effect of the chronic hypoxia persists for 5 weeks of normoxic recovery compared to the ischemic pre-conditioning (Neckar et al., 2004). Nevertheless, the adaptation to chronic hypoxia has not been studied as intensively as ischemic conditionings, therefore the molecular mechanisms responsible for the cardioprotective effect of chronic hypoxia are not still completely understood and need more research.

1.5 Hypoxia

The hypoxia is the result of disproportion between the oxygen supply and demand leading to the oxygen deficiency in tissues. According to the cause, four main types of hypoxia can be defined (reviewed in Ostadal and Kolar, 2007):

i) The histotoxic hypoxia represents a disability of tissue to utilize oxygen due to an injury caused by toxic substances, such as alcohol, cobalt, or cyanide, inhibiting the mitochondrial respiratory chain;

ii) The anemic hypoxia possesses the normal arterial pO_2 but has a decreased level of erythrocytes as well as hemoglobin or impaired function of the hemoglobin. This type

of hypoxia is found in anemia, poisoning by carbon dioxide, or in methemoglobinemia, resulting from an oxidation of iron in hemoglobin;

iii) The circulatory hypoxia has a decreased coronary blood flow leading to a lower oxygen as well as nutrients supply, despite the normal pO_2 and hemoglobin concentrations. The main cause of this hypoxia is the formation of atherosclerotic plaques, which can lead to the development of ischemia and in case of a complete cessation of coronary flow it may cause a myocardial infarction;

iv) The hypoxemic hypoxia is characterized by a low pO_2 in arterial blood under an adequate perfusion. This hypoxia, beside its relation to some cardiovascular diseases, also occurs naturally during perinatal development and during staying at high altitudes.

The adaptation to the high altitude hypoxia was shown to be a cardioprotective intervention relating to an acute form of ishemic heart disease, the myocardial infarction. It has been reported that populations living at high altitudes had a lower incidence of the myocardial infarction (Hurtado, 1960) and lower mortality rates of ischemic and coronary heart diseases (Mortimer et al., 1977; Voors and Johnson, 1979). Moreover, a reduced infarct size (Meerson et al., 1973; Turek et al., 1980), an improved recovery of the post-ischemic contractile function (Baker et al., 1997) and the hemodynamic function (Tajima et al., 1994), and a reduced incidence and severity of ischemic and reperfusion arrhythmias (Asemu et al., 2000; Meerson et al., 1987, 1989) in high altitude-residents have been observed. The cardiprotective effects of chronic hypoxia (CH) were also confirmed in experimental studies using a model of simulated hypoxia in a normobaric (Neckar et al., 2013) or hypobaric chamber (McGrath and Bullard, 1968; McGrath et al., 1973; Neckar et al., 2002a; b; Poupa et al., 1966; Widimsky et al., 1973). In contrast to protective effects of adaptation to CH, the study of Joyeux-Faure et al. (2005) showed that an extreme model of CH makes the heart more sensitive to the ischemic injury (Joyeux-Faure et al., 2005). And similarly, the study of Park and Suzuki (2007) showed that the I/R-induced myocardial injury depends on the duration of hypoxic exposure (Park and Suzuki, 2007). From these experimental data it is evident that the type of hypoxia, duration, intensity, and frequency of hypoxic episodes are critical factors determining whether hypoxia has beneficial or harmful effect (Beguin et al., 2005).

1.5.1 Experimental models of hypoxia

Experimental animals can be adapted to either the hypobaric or the normobaric hypoxia. The hypobaric hypoxia represents conditions with a low O_2 supply under a low barometric pressure, while the normobaric hypoxia demonstrates conditions with a low O_2 supply under a lower O_2 concentration (less than 21%). Both types of hypoxia are cardioprotective. However, whether their effects on cardiovascular parameters are similar or different it is still sporadic. While Sheedy et al. (1996) have found similarities between normobaric and hypobaric hypoxia actions (Sheedy et al., 1996), Savourey et al., (2003) have observed dissimilar effects of these two hypoxias (Savourey et al., 2003). Therefore, it cannot be excluded that these hypoxias will differ in the activation of molecular mechanisms due to the effect of the barometric pressure on O_2 supply into tissues.

According to the duration of hypoxia we can distinguish an acute and a chronic hypoxia. These two forms differ in the anti-arrhythmic and anti-necrotic effects. Neckar and his colleagues have observed that the adaptation to a normobaric hypoxia for hours or few days led to an activation of a strong anti-arrhythmic mechanism. The reduced incidence of ischemic arrhythmia persists into the fifth day of the adaptation. After this time, the anti-arrhythmic effect decreases and with increasing duration of the adaptation to hypoxia it disappears. In contrast, the anti-nectrotic mechanism, representing a reduced size of the necrotic tissue after induction of the myocardial infarction, is not visible in the first days of the adaptation to CH and it increases from the fifth day of the adaptation. It reaches its maximum at about the third or fourth week of the adaptation (Neckar et al., 2013).

The intensity of the hypoxia also plays an important role in the cardioprotection. The distinct barometric pressure during the adaptation to a hypobaric hypoxia can lead to the activation of different mechanisms. The adaptation to a hypobaric hypoxia simulated a high altitude 7000 m for 5 weeks still has a cardioprotective effect (Kolar et al., 2007). The question arises how long exposure would still results in the cardioprotection and whether the 7000 m is a limit altitude for the activity of survival mechanisms or not. For example, at the normobaric hypoxia, the adaptation to 10% O_2 possesses a cardioprotective effect (Neckar et al., 2013), but a lower concentration of O_2 can result in opposite impact (Joyeux-Faure et al., 2005).

The last critical factor determining whether hypoxia is beneficial or harmful is the frequency of hypoxic episodes. It has been observed that the intermittent normobaric hypoxia with 16 hours of daily reoxygenation was cardioprotective (*Figure 23*) (Kasparova et al., data in preparation), whereas the intermittent normobaric hypoxia with 1 hour of daily reoxygenation abolished this effect (Neckar et al., 2013). Conversely, Milano et al. (2010) have published that the daily reoxygenation decreased the myocardial injury and improved the post-ischemic recovery after chronic hypoxia (Milano et al., 2010a). Recent studies have reported the cardioprotective effect of the normobaric intermittent hypoxic conditioning, which is characterized by several short cyles of hypoxia per day (Mallet et al., 2006; Manukhina et al., 2013; Maslov et al., 2013).

In addition to the experimental models of the hypoxia, the sensitivity of the organism to the hypoxia has a vital role. Different species respond differently to the hypoxia. For example, cattle and pigs are more sensitive than rats and rabbits and these than dogs and sheep (Herget and Palecek, 1978; Reeves et al., 1979; Turek et al., 1975; Wauthy et al., 2004). Differences exist also between males and females. The female myocardium is more tolerant to the hypoxia than the myocardium of male (Ostadal et al., 1984b), which can be related to the effect of female sex hormones. And last but not least, the age affects the sensitivity of the organism to the hypoxia. La Padula and Costa (2005) have found that senescents rats (25 months) are more sensitive to the hypoxia than younger adult rats (up to 18 months), which may be associated with worse cardiovascular system plasticity (La Padula and Costa, 2005).

It can be generally concluded that the exposure to a moderate oxygen deprivation (physiological hypoxia) triggers defence mechanisms to deal with a reduced oxygen supply, and cardioprotective programmes. Conversely, a severe impairment of oxygen supply (pathophysiological hypoxia) may exceed the host organism's defence apparatus resulting in a maladaptive cardiac phenotype.

It should be also mentioned that beside the protective character, the adaptation to CH induces hypoxic pulmonary hypertension and right ventricle hyperthrophy, which, in case of an excessive hypoxic stimulus, may result in a congestive heart failure.

1.5.2 Adaptation to hypoxia

The adaptation to the hypoxia is associated with changes in the cardiac structure and function to preserve an adequate contractile function in spite of a lower oxygen concentration in blood. The oxygen sensing mechanisms and mechanisms maintaining the energy homeostasis play the important role in these adaptive processes. The reduced O_2 availability occurred during the hypoxia results in a reduced rate of electron transfer to O_2 by the complex IV, cytochrome *c* oxidase (COX). This impairs transfer between the complex III, cytochrome bc_1 complex, and the COX, which results in an increased rate of the ROS formation at the complex III. Interestingly, the continous hypoxia leads to an increased ROS production at the complex III (Chandel et al., 2000), while the intermittent hypoxia leads to an increased ROS generation at the complex I, NADH dehydrogenase, (Prabhakar et al., 2006). In both cases, the ROS function as signal molecules and activate HIF1 (Chandel et al., 2000; Guzy et al., 2005; Mansfield et al., 2005), the central regulator of cellular responses to chronic hypoxia (reviewed in Wang and Si, 2013). The HIF1 stimulates genes encoding proteins involved in adaptive mechanisms, which enables to cope with oxidative stress and relieve the severity of hypoxia.

1.5.2.1 Hypoxia inducible factor 1 (HIF1)

Mammalian tissues express three HIF isoforms, HIF1 (Semenza et al., 1991; Semenza and Wang, 1992), HIF2 (Drutel et al., 1996; Hirose et al., 1996), and HIF3 (Gu et al., 1998; Takahata et al., 1998), however, the most studied one is the HIF1. The HIF1 is composed of two subunit, α and β (Jiang et al., 1996; Wang and Semenza, 1995). The α subunit is localized in the cytosol (Kallio et al., 1998; Moroz et al., 2009) and is regulated by cytosolic hydrolylases, while the β subunit, identified as the aryl hydrocarbon nuclear translocator (ARNT) (Hoffman et al., 1991), is located in the nucleus (Chilov et al., 1999). The HIF1α activity is regulated by the concentration of oxygen (Cockman et al., 2000; Kamura et al., 2000; Ohh et al., 1998; Tanimoto et al., 2000; Wang et al., 1995a; b). Under normoxia (pO_2 = 0.21 bar), the HIF1α is hydroxylated at prolines by prolylhydrolylases (PHDs) (Bruegge et al., 2007; Fandrey et al., 2006; Gorres and Raines, 2010) and at asparagins by the asparagyl

hydroxylase, also called factor inhibiting HIF, FIH (Dann et al., 2002; Hewitson et al., 2002; Lando et al., 2002a; Linke et al., 2004; Lisy and Peet, 2008; Mahon et al., 2001; Stolze et al., 2004). Thus hydroxylated HIF1α is further ubiquitinylated by the proteasome complex and degraded (Kallio et al., 1999; Kamura et al., 2000). Under hypoxia ($pO_2 < 0.21$ bar), the PHDs and the FIH are inactivated (Lancaster et al., 2004; Lando et al., 2002b) and unhydroxylated HIF1α can enters the nucleus, where binds to its β subunit (Chilov et al., 1999) and co-activators (Arany et al., 1996; Carrero et al., 2000; Ema et al., 1999) and activates transcription of target genes. The HIF regulates the transcription of its target genes by binding to the hypoxia responsive element (HRE), which may be located either in the promoter region, or in distant regions called enhancers. There is a suggestion that target genes of the HIF could be up to 500 (Benita et al., 2009), but the evidence of a direct relationship has been described just for 80 of them (Benita et al., 2009; Schofield and Ratcliffe, 2004). The main target genes related to the adaptation to CH are summarized in the reviews of Semenza (Semenza, 2007, 2014) and the HIF1 action is illustrated on the *Figure 11* and described in the paper of Goda and Kanai (Goda and Kanai, 2012).

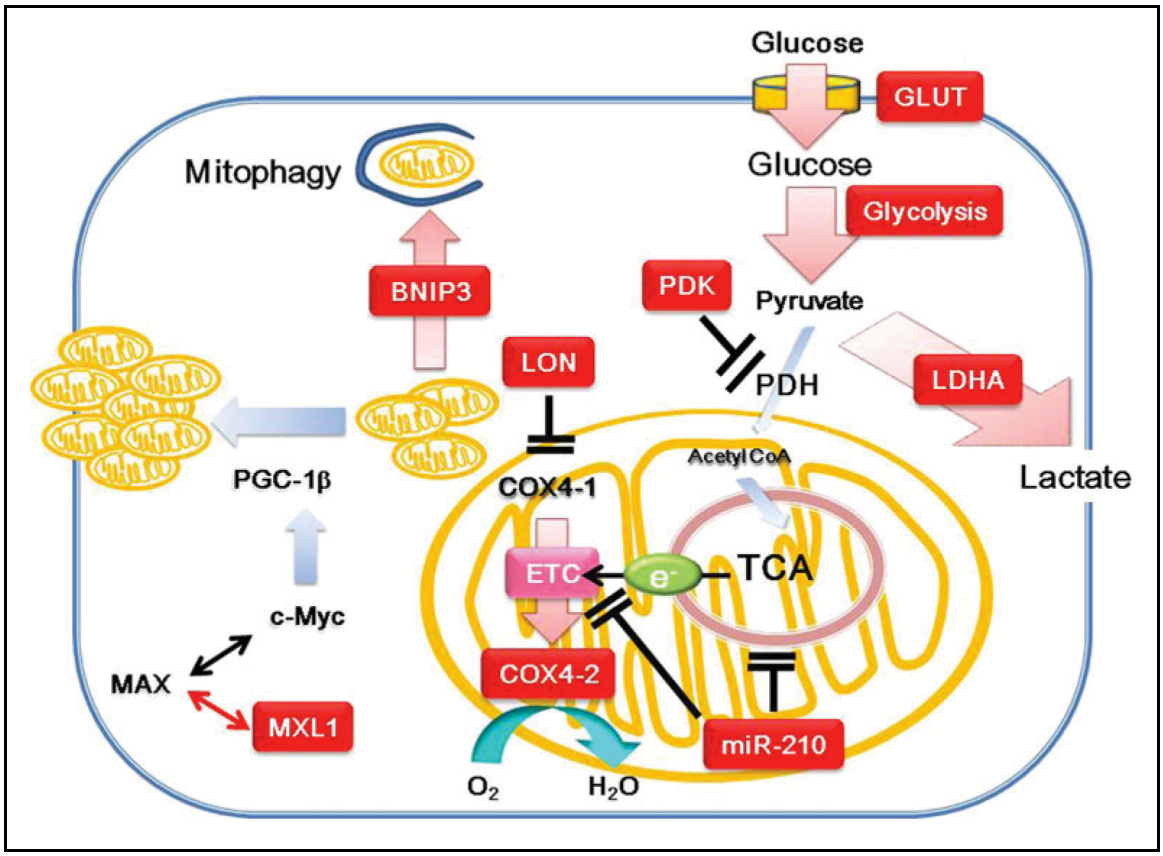

Figure 11: *The HIF1 action in cells. The GLUT, glucose transporter; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; Acetyl-CoA, acetyl conezym A; LDHA, lactate dehydrogenase A; LON, ATP-dependent protease in the mitochondrial matrix; COX4-1 and 4-2, cytochrome c oxidase subunit 4-1 and 4-2; ETC, electron transport chain; miR-210, micro RNA; TCA, tricarboxylic cycle; MXL1, max family protein; MAX, myc-associated factor X; BNIP3, member of the Bcl-2 family of mitochondrial proteins; PGC-1β, coactivator 1of peroxisome proliferator-activated receptor γ. The figure is reprinted from the paper Hypoxia-inducible factors and their roles in energy metabolism, Goda and Kanai, Springer and Int J Hematol (2012)95:457–463, Figure 1. The figure is republished with kind permission from Prof. Goda and Springer Science and Business Media.*

The hypoxia also activates other transcription factors, which cross-talk with the HIF1 to regulate cellular responses to hypoxia (Cummins and Taylor, 2005; Kenneth and Rocha, 2008). For example, the NFκB modulates the HIF1 transcription (Bonello et al., 2007; Jung et al., 2003; van Uden et al., 2008). The co-operation between the AP1 and the HIF1 has also been reported (Damert et al., 1997; Michiels et al., 2001). The p53 and the HIF1 regulate to each other. While the HIF1 activates the p53 (Chen et al., 2003), the p53 inhibits the HIF1 activity (Blagosklonny et al., 1998; Ravi et al., 2000). The Myc and HIF complexes can compete for binding sites at the promoters of target genes to alter their expression profile

(Koshiji et al., 2004). Beside transcription factors, the hypoxia controls transcription of target genes *via* an activation of the histone acetyltransferases or histone deacetylases and controls translation processes *via* activation of several kinases, which are responsible for the regulation of eukaryotic initiation factors (reviewed in Kenneth and Rocha, 2008).

1.5.2.2 Oxygen sensing mechanisms in hypoxia

From a physiological point of view, exposure to the hypoxia is initially associated with an increased respiratory rate and cardiac output, due to an increased adrenergic activity and elevated plasma concentration of catecholamines (Bao et al., 1997; Kumar et al., 2006; Ostadal et al., 1984a), to keep the adequate oxygen supply into tissues. The increased adrenergic activity results from the elevated carotid chemoreceptor response to a low arterial pO_2 during the hypoxia (Fletcher et al., 1992; Peng and Prabhakar, 2003; Rey et al., 2004) that is mediated by the ROS and the HIF1α signaling (Peng et al., 2006). It should be noted that with an acute exposure carotid chemoreceptors in humans are activated above a high altitude 2000 m, where the alveolar pO_2 is 60 mmHg and lower, while a chronic exposure can activate carotid chemoreceptors at lower altitudes. The carotid chemoreceptor sensor activity continuously increases with the length of the hypoxic exposure and reversibly decreases to a basal level during the normoxia (Peng et al., 2003). The initial increased cardiac output declines with the prolonged hypoxia to the sea-level values (Calbet, 2003). The increased sympathetic activity induced by the hypoxia also leads to an increase of blood pressure (reviewed in Fletcher, 2001; Prabhakar and Kumar, 2010). In addition, the adaptation to hypoxia elevates plasma levels of the vasoconstrictor endothelin 1 (Kanagy et al., 2001), which is also the HIF1α-target gene (Hu et al., 1998) and it increases a blood pressure *via* binding to its receptor (Allahdadi et al., 2008). The activation of the rennin-angiotensin system also contributes to an increased blood pressure *via* angiotensin II and its receptor (Fletcher et al., 2002). On the other hand, the HIF1 activates expression of genes and enzymes promoting relaxing the vascular tone (Wenger and Gassmann, 1997), such as the atrial natriuretic peptide, adrenomedullin (ADM), and NO synthase, which produces a known vasodilator NO, and thus decreases the blood pressure and peripheral resistance due to a vasodilation of arteries and

veins (Henley et al., 1992; Lyamina et al., 2011). Taken together, the effect of hypoxia on the cardiovascular system appears to be rather complex and further investigations are needed.

Other adaptive responses are represented by an increase of hematocrit (see *Table 1*, Chapter 3) and hemoglobin concentration *via* the HIF1 signaling pathway. The HIF1 activates the transcription of erythropoietin (EPO), which stimulates erythropoiesis (Jiang et al., 1996; Semenza and Wang, 1992), further the transcription of transferrin and its receptor (Lok and Ponka, 1999; Rolfs et al., 1997; Tacchini et al., 2002), which enhances the delivery of iron to the bone marrow for incorporation into hemoglobin, and also the transcription of heme oxygenase 1 (HO1) (Wang and Semenza, 1993; Wenger and Gassmann, 1997), which catalyzes a degradation of hem and release of iron needed for a erythropoiesis. The higher expression of HO1 under hypoxia has been already observed (Han et al., 2010). These mechanisms increase the blood oxygen transport capacity.

The HIF1 also regulates angiogenic processes *via* stimulation of the vascular endothelial growth factor (VEGF) and its receptor transcriptions, which mediates vascularization (Forsythe et al., 1996; Wenger and Gassmann, 1997) resulting in a higher density of coronary arteries and elevated coronary blood flow. The effect of hypoxia on the coronary blood flow is another controversial theme. Some authors have observed a greater coronary flow (Scheel et al., 1990; Turek et al., 1975) and increased capillary density (Miller and Hale, 1970; Zhong et al., 2002), while others have reported decreased capillary density (Clark and Smith, 1978; Smith and Clark, 1979) and low coronary flow (Grover and Alexander, 1971; Moret et al., 1972), or they have not found any changes in capillary density (Pietschmann and Bartels, 1985; Rakusan et al., 1981). These controversial findings observed across studies may be due to differences in the hypoxic model or among species. Rodents have relatively higher capacity for myocardial vascular growth than other mammals. Another important factor may be the age, since the degree of angiogenesis decreases with the age of animals (Tomanek et al., 2003).

1.5.2.3 Energy metabolism in hypoxia

The decreased level of oxygen slows down the mitochondrial oxidative phosphorylation *via* decreasing the ATP synthase activity (Nouette-Gaulain et al., 2005). Therefore it is necessary to activate mechanism that can produce the ATP even in the low oxygen. Another indicator of energetic status is PCr/ATP ratio, which is lower in hearts of people from high altitudes (Hochachka et al., 1996). These observations indicate an increased contribution of glucose to myocardial aerobic ATP production. The glucose and lactate are becoming the preferred substrates for the cardiac metabolism because of their higher efficiency in the ATP generation. The enhanced glucose uptake and utilization have been observed in people living in high altitudes or adapted to high altitudes and in rats adapted to the hypobaric hypoxia (Holden et al., 1995; Hurford et al., 1990; Roberts et al., 1996). The higher glucose transport into cardiomyocytes is mediated by the GLUT1 and GLUT4, which translocate into the plasma membrane during the hypoxia (Zhang et al., 1999). It has been also observed an increased expression of the GLUT1 (Sivitz et al., 1992) directly activated by the HIF1 (Wenger and Gassmann, 1997). The regulation of the glycolytic metabolism is mainly managed by the HIF1. The HIF1 activates expression of glycolytic enzymes (reviewed in

(Marin-Hernandez et al., 2009; Semenza et al., 1994, 1996), such as HK, G-6-P isomerase, PFK, aldolase, triose phosphate isomerase, GAPDH, PGK, phosphoglycerate mutase, enolase, and PK. The stimulated glycolysis leads to an increased production of lactate by the LDH, which is also an inducible target gene of the HIF1 (Firth et al., 1994, 1995; Wenger and Gassmann, 1997). The increased intracellular concentration of lactate activates the gene expression of the monocarboxylate transporter 1 (MCT1) and thus promotes lactate uptake (Hashimoto et al., 2007). The excessive lactate within cells is transported into blood by the MCT4, which is up-regulated during the hypoxia in the HIF-dependent manner (Ullah et al., 2006). This increased efflux of lactate from cardiomyocytes prevents further reduction of pH and development of lactic acidosis (McClelland and Brooks, 2002; Ullah et al., 2006). Despite the increased glycolytic rate, the FFAs are still metabolized, only the proportion between major cardiac substrates is changed. Under the normoxic conditions, the glucose uptake and glycolytic enzymes are inhibited by the FFA and their metabolites, while under hypoxia, the FFA uptake and utilization decreases, which leads to a decline in levels of FFA

metabolites and an activation of glycolysis. The hypoxia causes the decrease in the carnitine palmitoyltransferase 1 activity (Kennedy et al., 2001) *via* an increased malonyl-CoA concentration (Wang et al., 1996), which reduces the transport of the FFA into mitochondria. In addition, the hypoxia attenuates enzyme activities of the 3-hydroxyacyl-CoA-dehydrogenase (Bass et al., 1989; Daneshrad et al., 2000; Kennedy et al., 2001) and the medium-chain acyl-CoA dehydrogenase (Ngumbela et al., 2003), which decreases the FFA oxidation. The expression of β-oxidation enzymes is down-regulated by a reducing a level of their transcription factor the peroxisome proliferator-activated receptor (PPAR) α (Razeghi et al., 2001) mediated by the HIF1 (Narravula and Colgan, 2001). According to a lower ATP production, the cardiomyocytes switches into a slower mode by decreasing the contractility through the transition of myosin heavy chains from MyHCα to MyHCβ, which has a lower ATPase activity (Pissarek et al., 1997; Letout et al., 2005). The CK system also adapts to the hypoxic condition. The CKB isoform is up-regulated, which improves the PCr transfer within cells (Pissarek et al., 1997; Waskova-Arnostova et al., 2014). Generally, the adaptation to hypoxia leads to the activation of the fetal gene program.

1.5.2.4 Mitochondrial respiration in hypoxia

The HIF1 also regulates the mitochondrial metabolism. First, the HIF inhibits the PDH *via* activation of the PDC1 and thus inhibits the conversion of pyruvate to acetyl-CoA (Kim et al., 2006; Papandreou et al., 2006). The pyruvate is further converted into lactate by the LDH. The decreased level of the acetyl-CoA results in a decreased flux through the TCA and thereby reducing flux through the electron transport chain, which leads to a lower production of mitochondrial ROS. However, this mechanism does not have to be such effective in cardiomyocytes because of a high generation of the acetyl-CoA from the FFA oxidation, which is less, but still, active under hypoxic conditions.

The second way the HIF1 regulates the ROS over-production is the activation of switch in the COX from the COX4-1 subunit to the COX4-2 subunit that increases the efficiency of the complex IV (Fukuda et al., 2007).

The third posibility how to reduce the ROS production could be an activation of the mitochondrial autophagy. It has been recently observed, that patients with congenital heart

disease living at a high altitude resisted I/R injury during a cardiac surgery better than those at a low altitude, possibly through an elevated basal autophagy induced by the chronic hypoxia (Hu et al., 2014). The HIF1 activates the gene encoding BNIP3, a member of the Bcl-2 family of mitochondrial proteins, which triggers selective mitochondrial autophagy (Bellot et al., 2009; Zhang et al., 2008), even in cardiomyocytes (Regula et al., 2002). In addition, the HIF1 inhibits the mitochondrial biogenesis by repression of the c-Myc activity, which leads to an inhibition of the PPARγ coactivator 1 (PGC1) β, a mediator of mitochondrial biogenesis (Zhang et al., 2007). This is supported by a study, which reported a reduction in the mitochondrial oxidative capacity after exposure to hypobaric hypoxia (Green et al., 1989). The autophagy may be also induced by the AMPK without the HIF1 and BNIP3 participations, but this has been observed under severe oxygen deprivation (< 0.01% O_2) (Papandreou et al., 2008). On the contrary, there are also some evidences that hypoxia induces mitochondrial biogenesis in cardiac myocytes. The proliferation of smaller mitochondria has been found in response to chronic hypobaric hypoxia (Costa et al., 1988; Friedman et al., 1973). Hashimoto et al. (2007) has reported the association between a higher lactate concentration, which occurs during hypoxia, and enhanced expression of the PGC1α gene (Hashimoto et al., 2007). The recent study has found that the mitochondrial biogenesis is activated by the AKT kinase and endothelial NOS signaling pathway (Qin et al., 2014) and *via* activation of the PGC1α expression (Zhu et al., 2010). Another study has showed that the PGC1α expression is activated by the Ca^{2+}-calmodulin-dependent protein kinase (CaMK) (Wu et al., 2002a). The study of Ahuja et al. (2010) has demonstrated that the c-Myc activated the mitochondrial biogenesis but reduced the PGC1α level in the adult mouse heart (Ahuja et al., 2010). The elevated mitochondrial biogenesis has been also proposed by observed enhanced activities of respiratory enzymes and ATP synthesis in response to a high altitude hypoxia (Reynafarje and Marticorena, 2002). In addition, the PCr synthesis has been shown to be accelerated after adaptation to CH (Novel-Chate et al., 1995). These studies thus demonstrate how different models of hypoxia affect the mitochondrial metabolism and may result in distinct mitochondrial functional phenotypes. The activation of both autophagy and mitochondrial biogenesis processes may also be time dependent.

1.5.3 Cardioprotective mechanisms of hypoxia

The adaptive mechanisms activated during hypoxia may be potential candidates increasing a tolerance of the myocardium to I/R injury and arrhythmias. As the principal regulator of cellular responses to hypoxia is the HIF1, it may be assumed that the cardioprotective effects of hypoxia are mediated *via* the HIF1 signaling. The cardioprotective effect of the HIF1α has been demonstrated in numerous studies. The up-regulation and activation of the HIF1α using pharmacological agents, genetically modified animals, PHD inhibitors, or si-RNA mediated PHD gene silencing reduce the myocardial infarct size and improved the post-ischemic ventricular functions (Bao et al., 2010; Cai et al., 2003, 2008; Czibik et al., 2009; Eckle et al., 2008; Hyvärinen et al., 2010; Kido et al., 2005; Natarajan et al., 2006, 2007; Ockaili et al., 2005; Raphael et al., 2008; Xi et al., 2004). In most cases, the cardioprotective effect has been mediated by the HIF1α target genes, e.g. the HO1 (Ockaili et al., 2005), the ADM and the platelet-derived growth factor subunit B (Czibik et al., 2009), adenosine receptor (Eckle et al., 2008), or by the iNOS (Xi et al., 2004). However, the mechanism of the cardioprotective effect controlled by the HIF1α is not well understood. Cai et al. (2003) were the first to describe the cardioprotective effect of the HIF1α induced by an intermittent hypoxia using heterozygous knockout mice and suggest a potential role of the EPO (Cai et al., 2003). Recently, Wang and Si (2013) have shown the up-regulation of the HIF1α and the VEGF in rat hearts and suggested their participation in the cardioprotective effect of a short-term intermittent normobaric hypoxia (10% O_2 for 1, 7, 14, and 28 days) (Wang and Si, 2013). However, Forkel et al. (2004) have shown an increased mRNA of the HIF1α and the eNOS, but no improvement of the post-ischemic left ventricular function of rats adapted to a normobaric hypoxia (10.5% O_2 for 2 weeks) (Forkel et al., 2004). The study of Shi et al. (2002) described an increased association of the eNOS with the heat shock protein 90 in hearts of rabbits adapted to a normobaric hypoxia (12% O_2 for 9 days) and that this association helps to produce the NO and to limit the superoxide generation (Shi et al., 2002). On the other hand, the adaptation to an acute intermittent normobaric hypoxia (10% O_2 for 30 min, 2 h, or 4 h) induced the delayed cardioprotection in mice, which was triggered and mediated by the iNOS. This cardioprotective effect was abolished by the iNOS2 inhibitor (S-methylisothiourea) (Xi et al., 2002). The same findings have been reported by Beguin et al.

(2005), who observed that the infusion of a nonselective inhibitor of the NOS, the N(omega)-nitro-L-arginine methyl ester (L-NAME), into hearts of rats adapted to an acute intermittent normobaric hypoxia (5 or 10% O_2 for 30 min or 4 h) before the ischemia abolished the cardioprotective effect of the hypoxic adaptation (Beguin et al., 2005). The contribution of the iNOS in the protection of rat hearts against I/R injury has been also observed after the adaptation to an intermittent hypobaric hypoxia (5000 m for 42 days) using the iNOS-selective inhibitor aminoguanidine (Ding et al., 2005). Other studies on rabbits using a model of a normobaric continuous hypoxia (12% O_2 for 7-10 days) and the L-NAME inhibitor has also reported that the enhanced NOS activity is associated with a resistance to myocardial ischemia (Baker et al., 1999; Fitzpatrick et al., 2005). It has recently been suggested that the ROS/NO balance plays an important role in cardioprotective mechanisms. The adaptation of rats to a sub-chronic hypobaric hypoxia (7620 m for 5 days) significantly increased the NO level and enzyme activities of antioxidants and simultaneously markedly decreased the level of ROS (Singh et al., 2013). The cardioprotective effect of antioxidant enzymes has been already described for guinea pigs adapted to a chronic intermittent hypobaric hypoxia (5000 m for 28 days) (Guo et al., 2009). These authors have found that the pre-treatment of control hearts with an antioxidant mixture containing the superoxide dismutase (SOD) and the catalase (CAT) caused cardioprotective effects similar to the hypoxic adaptation and that the irreversible CAT inhibitor aminotriazole abolished the cardioprotection of the hypoxia (Guo et al., 2009). Based on all these observations, the regulation of the ROS over-production and the enhancement of antioxidant capacity represent promising cardioprotective targets for clinical studies.

Beside the antioxidant defense, the NO also plays a role in the regulation of a blood pressure *via* vasodilation of vessels (Archer et al., 1994). The angiotensin II and its receptor represent other important regulators of the blood pressure. It has been demonstrated that the inhibition of the angiotensin II receptor by the candesartan exhibited the cardioprotection of a chronic continuous normobaric hypoxia (10% O_2 for 2 weeks) in part through the reduction of the blood pressure and cytokine expression in diabetic rats (Inamoto et al., 2006). In addition, the angiotensin II receptor 1 antagonist irbesartan completely abolished the improvement of the post-ischemic recovery of the cardiac contractile function during the reperfusion. Thus, the angiotensin II receptor 1 pathway plays an important role in the coronary angiogenesis and

improved the cardiac ischemic tolerance induced in neonatal rats by a chronic intermittent hypobaric hypoxia (5000 m for 10 days) (Rakusan et al., 2007).

In addition to the ROS-mediated cell and tissue damage, the Ca^{2+} overload induces the apoptosis and inflammation. Therefore, the regulation of processes maintaining the Ca^{2+} homeostasis could be another cardioprotective tool against I/R injury. It has been demonstrated that the CaMKII is involved in the cardioprotection induced by the adaptation of rats to an intermittent hypobaric hypoxia (5000 m for 42 days) due to an administration of the CaMKII inhibitor, the KN-93, which significantly attenuates the protective effect of mentioned hypoxia (Xie et al., 2004; Yu et al., 2009). The intermittent hypobaric hypoxia (5000 m for 42 days) has also up-regulated the phospholamban phosphorylation site for the CaMKII as well as for the PKA, which may consequently contribute to the cardioprotection of rat hearts against the I/R injury (Xie et al., 2005). The elevated CaMKII inhibits the phospholamban *via* phosphorylation and thus activates the SERCA and the Ca^{2+} re-uptake (Mattiazzi and Kranias, 2014). Another study reported that the intermittent hypobaric hypoxia (5000 m for 42 days) may preserve the Ca^{2+} homeostasis and contraction by preserving the ryanodine receptors (RyRs) and the SERCA2 proteins as well as the NCX activity in isolated rat cardiomyocytes during the I/R injury (Chen et al., 2006). The following study has demonstrated that also an intermittent normobaric hypoxia (10% O_2 for 3, 7, and 14 days) confers the cardioprotection against the I/R injury in rat cardiomyocytes by altered the Ca^{2+} handling with augmented the RyR and the NCX activities *via* the PKA and PKC, but not *via* the CaKMII activation (Yeung et al., 2007).

The high intracellular concentration of Ca^{2+} leads to an opening of the MPT pore and to the subsequent apoptosis. Targeting the MPT pore and other mitochondrial pro- and anti-apoptotic proteins may be significant protective mechanisms against the cell death. Zhu et al. (2006) have demonstrated that the inhibition of the MPTP opening, inducing by an intermittent hypobaric hypoxia (5000 m for 42 days), attenuated the intracellular as well as the mitochondrial Ca^{2+} overloading in rat cardiomyocytes, which contributed to the cardioprotection of this hypoxic adaptation. On the other hand, opening of the MPT pore with the atractyloside immediately at the reperfusion abolished these cardioprotective effects (Zhu et al., 2006). Moreover, the adaptation of rats to an intermittent hypobaric hypoxia (5000 m for 42 days) attenuated the I/R-induced apoptosis *via* increasing the ratio of Bcl-2/BAX,

especially in the membrane fraction (Dong et al., 2003). Recent study of Neckar et al. (2013) has reported the cardioprotective effect of a chronic continuous normobaric hypoxia (10% O_2 for 15 and 30 days) *via* the activation of the mitochondrial Ca^{2+}-activated K^+ (BKCa) channels. Authors have demonstrated that the using of the BKCa channel blocker paxilline attenuated the cytoprotective effect in cardiomyocytes isolated from hypoxic rat hearts (Neckar et al., 2013).

Other ionic channels regulating the distribution of Ca^{2+} are the K_{ATP} channels. They control the membrane potentials, regulate membrane swelling, and maintain the optimal sensitivity to sympathetic signals. It has been observed that the sarcolemmal and mitochondrial K_{ATP} channels contribute to the cardioprotection in rabbit hearts adapted to a normobaric continuous hypoxia (12% O_2 for 8-10 days). The activation of the K_{ATP} channels is associated with the resistance of rabbit hearts adapted to a normobaric continuous hypoxia (12% O_2 for 7-10 days) to the myocardial ischemia. The application of a general K_{ATP} channel blocker glibenclamide (Baker et al., 1997; Fitzpatrick et al., 2005) or a selective mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate and a sarcolemmal K_{ATP} channel blocker HMR 1098 abolished cardioprotection in previously hypoxic hearts (Kong et al., 2001). On the other hand, the application of K_{ATP} channel agonist bimakalim increased the recovery of the left ventricular developed pressure in normoxic rabbit hearts (Baker et al., 1997). The K_{ATP} channels are also associated with a resistance of rat hearts to the I/R injury induced by the adaptation to an intermittent hypoxia (5000 m for 42 days) (Zhu et al., 2003). Authors reported that the glibenclamide as well as the 5-hydroxydecanoate abolished the cardioprotective and cytoprotective effects of the hypoxic adaptation, whereas the K_{ATP} channel opener, pinacidil, attenuated the Ca^{2+} overloading during the ischemia and reperfusion in isolated normoxic cardiomyocytes. However, Asemu et al. (1999) first proposed that the mitochondrial K_{ATP} channel, rather than the sarcolemmal K_{ATP} channel, appears to be involved in the protective mechanism afforded by the adaptation to an intermittent hypobaric hypoxia (5000 m for 2-3 weeks or 5-6 weeks) (Asemu et al., 1999). They have observed an increased number of arrhythmias in normoxic as well as hypoxic rat hearts using a glibenclamide and in contrast, a decreased number of arrhythmias in the normoxic hearts using a diazoxide, the mitochondrial K_{ATP} channel opener. However, Suzuki et al. (2003) have reported that the diazoxide activated the sarcolemmal K_{ATP} channels, not the mitochondrial K_{ATP} channels in mouse hearts (Suzuki

et al., 2003). It seems that the selective K_{ATP} channel modulators might not be sufficiently selective under certain conditions and might be species-dependent. Results similar to those of Asemu et al. (1999) have been later demonstrated by Kolar et al. (2005), who have used a novel agent MCC-134, which inhibits the mitochondrial K_{ATP} and activates the sarcolemmal K_{ATP} channels, and thus found that the opening of the mitochondrial K_{ATP} channels but not the sarcolemmal K_{ATP} channels plays a crucial role in the mechanism by which the intermittent hypobaric hypoxia (7000 m, 5-6 weeks) improves the cardiac tolerance to the I/R injury in rats (Kolar et al., 2005). The cardioprotective effect of the mitochondrial K_{ATP} channels using a 5-hydroxydecanoate inhibitor has been also confirmed for the intermittent hypobaric hypoxia (5000 m for 24-32 days or 6 weeks) (Neckar et al., 2002b; Xie et al., 2004). The mitochondrial K_{ATP} channels also underlie cardioprotective mechanisms in rat hearts induced by the adaptation to an acute intermittent normobaric hypoxia (5 or 10% O_2 for 30 min or 4 h) (Beguin et al., 2005), as well as in rabbit hearts induced by the adaptation to a chronic continuous normobaric hypoxia (12% O_2 for 7-10 days). This improves the mitochondrial bioenergetics *via* regulation of the ATP synthesis rate (Eells et al., 2000). It has been recently reported that the inhibition of the mitochondrial ATP synthase by the oligomycin abolished the improvements of the post-ischemic recovery of the left ventricle function, mitochondrial membrane potential, and respiratory control ratios in rats previously adapted to an intermittent hypobaric hypoxia (5000 m for 4 weeks) (Wang et al., 2012).

The high ATP/ADP ratio is essential for the activity of ATPases. The Na^+/K^+ ATPase is one of the main consumers of the ATP (Rolfe and Brown, 1997). Therefore, any change in the production of the ATP has a significant impact on its function. Under the ischemia, when the ATP synthesis is strongly inhibited, the function of the Na^+/K^+ ATPase is ceased, thereby exacerbating the intracellular Na^+ overload (Chapter 1.4). Guo et al. (2011) have shown that the enhancement of the Na^+/K^+ ATPase activity by a chronic intermittent hypobaric hypoxia (5000 m for 28 days) protected guinea pig hearts against the I/R injury, while the inhibition of the Na^+/K^+ ATPase activity by the oubain attenuated this protective effect (Guo et al., 2011).

It is generally accepted that the adaptation to a hypoxia increased the level of catecholamines in blood resulting in the adrenergic stimulation (Bao et al., 1997; Kumar et al., 2006; Ostadal et al., 1984a). Activation of the α_{1B}-adrenergic receptors by its agonist phenylephrine has been shown to improved the post-ischemic myocardial performance in rats

adapted to an intermittent hypobaric hypoxia (5000 m for 4 weeks) *via* the PKC-ε signaling pathway and inhibiting the matrix metalloproteinase 2, which is involved in the breakdown of extracellular matrix proteins (Gao et al., 2014). Moreover, the α_{1B}-adrenergic receptor is the target gene of the HIF1α (Eckhart et al., 1997). Other study by Mallet et al. (2006) has demonstrated that the episodic β_1-adrenergic activation during an intermittent hypoxic conditioning (9.5-10% O_2, 20 days) evoked a progressive development of the powerful resistance to the myocardial ischemia in dogs (Mallet et al., 2006). Beside the adrenergic activity, activation of opioids receptors by elevated levels of endogenous opioid peptides is responsible for the cardioprotection induced by the adaptation of rats to a chronic continuous normobaric hypoxia (12% O_2 for 3 weeks) (Maslov et al., 2013).

The adrenergic and other extracellular signals activate a variety of signaling cascades, whose central regulators are various protein kinases. It has been reported that the administration of the PI3K inhibitor wartmaninn in mice adapted to an intermittent normobaric hypoxia (6-8% O_2 for 14 days) reduced also the level of the phospho-AKT and magnified the infarct size, indicating that the PI3K/AKT pathway is crucial for the cardioprotection induced by the adaptation to a hypoxia (Milano et al., 2013). It has been also described that the PI3K/AKT pathway have an important role in the cardioprotection mediated by a chronic intermittent normobaric hypoxia (10% O_2) with 1 h daily reoxygenation. Moreover, the extracellular signal-regulated kinases 1/2 (ERK1/2) are also involved in the cardioprotective mechanisms of this type of hypoxia (Milano et al., 2010b). The recent study has reported that the cardioprotection induced by the adaptation of rats to an intermittent hypobaric hypoxia (5000 m for 6 weeks) is mediated by the ERK1/2 signaling, which was found in H9C2 cells using an ERK1/2 phosphorylation inhibitor PD98059 (Meng et al., 2014). The PKC represents the other kinase playing a role in cardioprotective mechanisms mediated by the adaptation of rats to an intermittent hypobaric hypoxia (5000 m for 42 days) (Ding et al., 2004). These authors have found that a PKC antagonist chelerythrine significantly inhibited the protective effects of the intermittent hypobaric hypoxia and that the PKC contributed to the elimination of Ca^{2+} and Na^{+} overloads in isolated hypoxic cardiomyocytes. This might underlie the mechanism of the cardiprotection. Neckar et al. (2005) have described that the chronic intermittent hypobaric hypoxia (7000 m for 5-6 weeks) induces the cardioprotection in the rat myocardium, which is partially mediated by the PKC-δ. The administration of a selective

PKC-δ inhibitor, rottlerin, attenuated the infarct size-limiting effect of a chronic hypoxia (Neckar et al., 2005). A later study from the same laboratory demonstrated that the infarct size-limiting mechanism of this intermittent hypobaric hypoxia mediated by the PKC-δ-dependent pathway does not apparently involve the increased capacity of major antioxidant enzymes (Kolar et al., 2007). Wang et al. (2011) have found that the PKC-ε and AKT pathways may form a positive feedback loop. Both kinases mediate the ROS-dependent cardioprotection in rat hearts during the early reperfusion period induced by an intermittent hypobaric hypoxia (5000 m for 4 weeks), because the inhibition of the AKT with the wortmannin and the PKC-ε with the εV1-2 abrogated the intermittent hypobaric hypoxia-improved post-ischemic left ventricle performance (Wang et al., 2011). Other study, using known inhibitors wortmannin, chelerythrine, PD98059, and a selective inhibitor of the p38 mitogen-activated protein kinases (p38 MAPK), SB203580, has reported that not the PI3K, but the PKC, ERK1/2, and p38 MAPK are involved in the cardioprotective effects induced by the adaptation of rats to an acute intermittent normobaric hypoxia (10% O_2 for 4 h) (Beguin et al., 2007). In addition to the cardiprotective role of the PKC-ε and p38 MAPK, the activation of the JUN kinase signal transduction pathway may be also responsible for the cardioprotection in the chronically hypoxic rabbit hearts due to a fact that the inhibition of the JUN kinase with the curcumin abolished this cardioprotective effect (Rafiee et al., 2002).

Given the existence of other cardioprotective mechanisms activated under different conditions than under the adaptation to a hypoxia, it can be assumed that more signaling pathways and molecules maintaining energy and ion homeostasis and preventing an excessive production of the ROS and the Ca^{2+} overload might be involved in the cardioprotection induced by a hypoxia.

2. AIMS OF THE THESIS

2.1 Study 1: The comparison of the left (LV) and right (RV) ventricles under normoxia

The specific objectives of Study 1 were:

- To determine the CK and HK isoform expressions, the total CK and HK enzyme activities, and the co-localization of HK isoforms with mitochondria.
- To examine the phoshorylated and non-phosphorylated AKT protein levels and the phoshorylated/non-phosphorylated AKT ratios.

2.2 Study 2: The effect of the normobaric hypoxia and I/R insult on the CK and HK enzymes

The specific objectives of Study 2 were:

- To determine the CK and HK isoform expressions and the total CK and HK enzyme activities in both ventricles of rats adapted to protective and non-protective regimens of moderate normobaric hypoxia.
- To investigate the co-localization of HK isoforms with mitochondria in the LV as well as in the RV of rats adapted to a protective regimen, continuous normobaric hypoxia.
- To examine the effect of I/R insult on the HK isoform expressions and on the total CK and HK enzyme activities in the LV of rats adapted to a protective regimen, continuous normobaric hypoxia.

2.3 Study 3: The effect of the hypobaric hypoxia on the CK and HK enzymes

The specific objectives of Study 3 were:

- To determine the CK and HK isoform expressions, the total CK and HK enzyme activities, and the co-localization of HK isoforms with mitochondria in the LV as well as in the RV of rats adapted to a severe intermittent hypobaric hypoxia.

3. METHODS

3.1 Animals

Adult male Wistar rats were obtained from breeding companies Velaz, Ltd., Czech Republic (Chapter 4, Chapter 5, and Chapter 6) and Charles River Laboratories, Inc., Germany (Chapter 5 and Chapter 6). Animals were fed a standard laboratory diet and kept at a 12/12-h light/dark cycle. The maintenance and handling of the experimental animals were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic.

3.2 Adaptation to normobaric hypoxia

Animals were housed in a normobaric chamber equipped with hypoxic generators (Everest Summit, Hypoxico Inc., NY, USA), which reduced the percentage of oxygen in the ambient air to 10%, corresponding to a high altitude of 5500 m. Animals were exposed to a normobaric hypoxia for 3 weeks under the following three regimens (*Figure 12*): the continuous hypoxia for 24 h/day (CNH), the intermittent hypoxia for 23 h/day with a single 1-h normoxic period per day (INH-23), and the intermittent hypoxia for 8 h/day with a single 16-h normoxic period per day (INH-8). The control group (N) was kept under normoxic conditions for the same period of time. Animals had a free access to the water and standard diet for the whole time of experiments.

Figure 12: *The scheme of the adaptation to the chronic normobaric hypoxia. The N, normoxia; INH-8, intermittent hypoxia for 8 h/day with a single 16-h normoxic period per day; INH-23, intermittent hypoxia for 23 h/day with a single 1-h normoxic period per day; CNH, continuous hypoxia for 24 h/day.*

3.3 Adaptation to hypobaric hypoxia

Animals were housed in a hypobaric chamber, in which they were acclimated to a reduced barometric pressure for 2 weeks to reach a barometric pressure (PB), which corresponds to a high altitude of 7000 m (PB = 308 mm Hg, 41 kPa; pO_2 = 65 mm Hg, 8.6 kPa) (*Figure 13*). Thereafter, animals were exposed to a hypobaric hypoxia simulated a high altitude of 7000 m for 3 weeks. Animals were exposed to a hypobaric hypoxia intermittently for 8 hours a day (IHH-8) (*Figure 14*). The control group (N) was kept under normoxic conditions for the same period of time at PB and pO_2 equivalent to an altitude of 200 m (PB = 742 mm Hg, 99 kPa; pO_2 = 155 mm Hg, 20.7 kPa). Animals had a free access to the water and standard diet for the whole time of experiments.

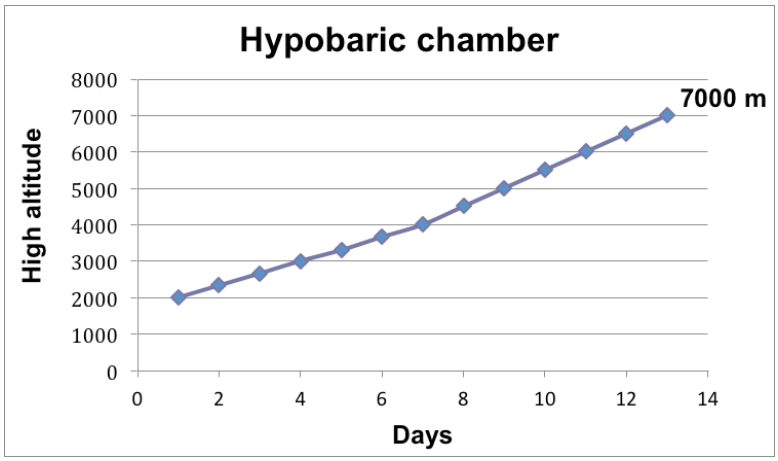

Figure 13: *The illustration of the barometric pressure reduction during the adaptation to the intermittent hypobaric hypoxia. The continuous acclimatization was performed for 2 weeks to reach a high altitude of 7000 m.*

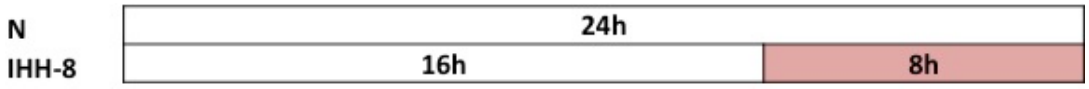

Figure 14: *The scheme of the adaptation to the intermittent hypobaric hypoxia. The N, normoxia; IHH-8, intermittent hypoxia for 8 h/day with a single 16-h normoxic period per day.*

3.4 Ischemia-reperfusion protocol

The normoxic rats and rats adapted to the CNH regimen were killed by a cervical dislocation. Hearts were rapidly removed, placed on the Langendorff apparatus and perfused by Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, pH 7.4) saturated by 95% O_2 and 5% CO_2 at the temperature 37°C and a constant pressure (100 cm H_2O). The Langendorff hearts were divided into 3 groups: i) hearts stabilized for 15 min by perfusion (KN, KH); ii) hearts stabilized for 15 min and subjected to a no-flow ischemia for 10 min (IN, IH); iii) hearts stabilized for 15 min, subjected to ischemia for 10 min and then subjected to reperfusion for 10 min (IRN, IRH). After the start of reperfusion, the effluent samples (0.5 ml) were collected at intervals: 10 s, 20 s, 30 s, 60 s, 3 min, and 10 min to determine the LDH release as an indicator of degree of cell damage. The scheme of I/R protocol is shown in *Figure 15*. And the end of each period, hearts were removed from the apparatus, separated into LV, RV, and septum (S), frozen in liquid nitrogen, weighed (*Table 1*), and stored for further analyses.

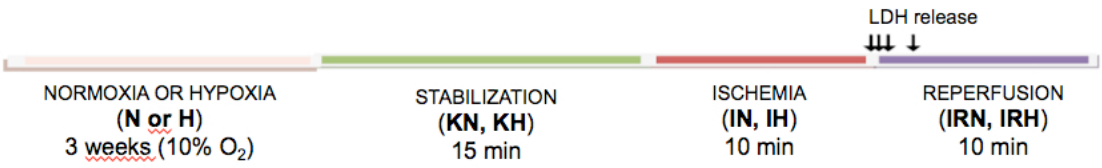

Figure 15: *The scheme of the I/R protocol. The N, normoxic rats; H, rats adapted to continuous normobaric hypoxia; KN, stabilized normoxic hearts; KH, stabilized hypoxic hearts; IN, ischemic normoxic hearts; IH, ischemic hypoxic hearts; IRN, normoxic I/R hearts; IRH, hypoxic I/R hearts; LDH, lactate dehydrogenase.*

Table 1: *The weight parameters of experimental animals. The N, normoxia; INH-8, intermittent normobaric hypoxia for 8 h/day; INH-23, intermittent normobaric hypoxia for 23 h/day; CNH, continuous normobaric hypoxia; IHH-8, intermittent hypobaric hypoxia for 8 h/day; KN, perfused normoxic hearts; IN, , perfused normoxic hearts subjected to ischemia; IRN, perfused normoxic hearts subjected to I/R insult; KH, perfused hypoxic hearts; IH, perfused hypoxic hearts subjected to ischemia; IRH, perfused hypoxic hearts subjected to I/R insult; BW, body weight; RV, weight of the right ventricle (RV); LV, weight of the left ventricle (LV); S, weight of the septum; RV/BW, relative weight of the RV; LV/BW, relative weight of the LV; HW/BW, relative weight of the heart. Values are mean ± S.E.M.*P<0.05 vs. N, *P<0.05 vs. corresponding normoxic group, $P<0.05 vs. KN.*

Velaz, Ltd., Czech Republic

Group	BW (g)	RV (mg)	LV (mg)	S (mg)	RV/BW (mg/g)	LV/BW (mg/g)	HW/BW (mg/g)	Hematocrit (%)
N	361 ± 9	189 ± 5	460 ± 14	201 ± 6	0.52 ± 0.01	1.28 ± 0.04	2.36 ± 0.05	42.9 ± 0.9
INH-8	347 ± 5	224 ± 6*	472 ± 9	194 ± 3	0.65 ± 0.02*	1.36 ± 0.03	2.57 ± 0.06	54.2 ± 1.3*
INH-23	308 ± 8*	319 ± 16*	453 ± 21	178 ± 10	1.03 ± 0.04*	1.47 ± 0.05	3.08 ± 0.10*	58.5 ± 1.7*
CNH	311 ± 7*	351 ± 28*	451 ± 26	189 ± 12	1.12 ± 0.07*	1.45 ± 0.08	3.18 ± 0.15*	61.5 ± 1.5*
N	425 ± 9	203 ± 8	539 ± 15	244 ± 11	0.48 ± 0.01	1.27 ± 0.03	2.32 ± 0.04	42.6 ± 1.4
IHH-8	371 ± 6*	293 ± 8*	624 ± 27*	235 ± 7	0.79 ± 0.02*	1.68 ± 0.07*	3.10 ± 0.09*	59.7 ± 2.0*

Charles River Laboratories, Inc., Germany

Group	BW (g)	RV (mg)	LV (mg)	S (mg)	RV/BW (mg/g)	LV/BW (mg/g)	HW/BW (mg/g)
N	423 ± 3	246 ± 6	528 ± 16	254 ± 13	0.58 ± 0.01	1.25 ± 0.03	2.43 ± 0.06
IHH-8	375 ± 8*	391 ± 29*	617 ± 32	254 ± 22	1.04 ± 0.08*	1.65 ± 0.1*	3.37 ± 0.21*
KN	393 ± 4	230 ± 6	684 ± 14	240 ± 8	0.59 ± 0.02	1.74 ± 0.05	2.94 ± 0.04
IN	390 ± 8	255 ± 10	778 ± 18 $	272 ± 7	0.65 ± 0.02	2.00 ± 0.03	3.35 ± 0.03
IRN	380 ± 10	242 ± 16	645 ± 14	234 ± 9	0.63 ± 0.03	1.71 ± 0.06	2.96 ± 0.05
KH	354 ± 8*	454 ± 34*	682 ± 25	293 ± 10*	1.28 ± 0.08*	1.93 ± 0.07	4.04 ± 0.16*
IH	335 ± 3*	476 ± 43*	724 ± 25	291 ± 13	1.42 ± 0.12*	2.16 ± 0.07	4.45 ± 0.19*
IRH	350 ± 4	392 ± 15*	651 ± 18	273 ± 9	1.12 ± 0.05*	1.86 ± 0.05	3.76 ± 0.09*

3.5 Tissue preparation

All rats were sacrificed by decapitation and the hearts were rapidly excised and washed in ice-cold saline. The LV and RV were dissected, immediately frozen in liquid nitrogen and weighed. Frozen tissue pieces were pulverized in liquid nitrogen and subsequently homogenized either in homogenization buffer in the ratio 1:8 (w/v) for Western blot (WB) and enzyme analyses or in TRIZOL Reagent (Invitrogen-Molecular Probes, Eugene, OR) for isolation of RNA. The homogenization buffer (pH 7.4) contained 12.5 mM TRIS, 2.5 mM EGTA, 1 mM EDTA, 250 mM sucrose, 5 mM DL-Dithiothreitol (DTT), and protease inhibitor cocktail (cOMPLETE, Roche Diagnostics). The homogenization buffer for analyses of phosphorylated AKT (Chapter 4) also included phosphatase inhibitor cocktail (PhosSTOP, Roche Diagnostics) to preserve the phosphorylation state. The 100 µl of homogenates were separated, aliquoted and stored at -80°C for enzyme analyses. The rest of homogenates were diluted 1:1 (v/v) with buffer containing 5 M urea, 2 M thiourea, 10 mM sodium pyrophosphate tetrabasic decahydrate, and 0.13% 2-mercaptoethanol, which enables a better solubilization of contractile proteins. Thereafter, homogenates with urea were diluted with a 4x concentrated sample loading buffer (260 mM TRIS/HCl pH 6.8, 40% glycerol, 8% SDS, 8% DTT, 0.04% bromophenol blue), up to concentration 3 µg/µl, aliquoted and stored at -80°C for WB analyses. Protein concentration was measured in both original homogenates and homogenates with urea using the Bradford dye binding assay (Sigma-Aldrich).

3.6 RNA isolation and Real-Time quantitative RT-PCR analysis

The total cellular RNA was extracted from each left and right ventricles using TRIZOL Reagent and cleaned up by DNA-free RNA kit (Ambion, Carlsbad, CA). The purity and integrity of the RNA preparations was checked using NanoDrop spectrophotometer and by agarose gel electrophoresis. The total RNA was converted to cDNA using a RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas UAB, Vilnius, Lithuania) with oligo(dT) primers according to the manufacturer's instructions. Real-time PCR was performed on a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) using the mono color hydrolysis probe method (Roche Applied Sciences) with the appropriate

Probe Master kit (Roche Applied Sciences) according to the manufacturer's protocol (Chapter 5 and Chapter 6) or using SyberGreen protocol with the SyberGreen Master Mix (Bio-Rad) according to the manufacturer's instructions (Chapter 4). Specific primers and probes for HK1, HK2, CKM, CKB, mtCKs, and the reference gene hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) were designed using the Universal Probe Library Assay Design Center (Roche Applied Science). Sequences of the primers are listed in *Table 2*. PCR amplification was performed under following conditions: initial denaturation at 95°C for 10 min, followed by 50 cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 10 s where fluorescence was acquired, and elongation at 72°C for 5 s. The data used for the calculations are the mean of the crossing point (CP) values obtained from qPCR performed in triplicates. The variation between triplicate determinations did not exceed 0.5 CP. Melting curve analysis was performed to ascertain the presence of a single amplicon for each pair of primers. Standard curves were generated for each pair of primers using a 3-fold serial dilution of cDNA. The amplification efficiency of the PCR reaction for each primer pair was then calculated from the standard curve in order to estimate precisely the relative transcript expression. Transcript levels were normalized to the level of the reference gene HPRT1 transcript. High expression stability of HPRT1 had been established previously (Bohuslavova et al., 2010). The expression level of mRNA was normalized with regard to specific PCR efficiency (E) for each gene according to the following formula (Pfaffl, 2001):

$$\text{Normalized amount} = (1+E)^{\text{CT reference transcript}} / (1+E)^{\text{CT target transcript}}$$

No-template and no-RT control reactions were performed to screen for false amplification and to confirm the absence of DNA contamination.

Table 2: *The specific primers designed using the Universal Probe Library (UPL) Assay Design Center (Roche Applied Science). The HK, hexokinase; CKM, creatine kinase muscle; CKB, creatine kinase brain; mtCKs, mitochondrial creatine kinase sarcomeric; HPRT1, hypoxanthine-guanine phosphoribosyl transferase1.*

Transcript	Forward primer	Reverse primer	UPL probe number
HK1	tctgggcttcaccttctcat	atcaagattccacagtccaggt	121
HK2	ccagcagaacagcctagacc	agatgccttgaatccctttg	101
CKM	ccgcagcatcaagggtta	cccgtcaggctgttgaga	16
CKB	ccacttcctcttcgacaagc	ggaacgtcttattgtcattgtgc	84
mtCKs	gccacccccttcattaagactg	caaaaaggtcagcaaacacct	83
HPRT1	gaccggttctgtcatgtcg	acctggttcatcatcactaatcac	95

3.7 SDS-PAGE and Western blot analysis

Individual homogenates from the LV and RV samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide separating gels and 5% polyacrylamide stacking gels at constant voltage of 200 V and room temperature (Mini-PROTEAN TetraCell, Bio-Rad). The gel-resolved proteins were electrotransferred onto the nitrocellulose membrane (0.2 μm pore size, Protran BA 83, Whatman, Germany) at constant voltage of 100 V and 350 mA current for 1 h at 4°C (Mini Trans-Blot, Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in TRIS-buffered saline solution (TBS) containing Tween 20 (TTBS). After washing in TTBS (3x15min), membranes were incubated overnight at 4°C with the primary polyclonal antibodies (*Table 3*).

Table 3: *The specific primary antibodies for the Western blot (WB) and immunofluorescence staining (IF). The HK, hexokinase; CKM, creatine kinase muscle; CKB, creatine kinase brain; mtCKs, mitochondrial creatine kinase sarcomeric; AKT, AKT kinase; pS-AKT, phosphorylated AKT on serin 473; pT-AKT, phosphorylated AKT on threonin 308; OXPHOS, compartments of the mitochondrial respiratory chain and ATP synthase; GAPDH, glycerlaldehyde 3-phosphate dehydrogenase; Bcl-2, anti-apoptotic protein from the Bcl-2 protein family; BAX, pro-apoptotic protein from the Bcl-2 protein family.*

Protein	Primary antibody	Company	Specific use	Dilution
HK1	sc-28885	Santa Cruz Biotechnology	WB, IF	1:2000, 1:50
	ab78420	Abcam	WB, IF	1:1000, 1:50
HK2	sc-28889	Santa Cruz Biotechnology	WB, IF	1:800, 1:50
	ab78259	Abcam	WB, IF	1:1000, 1:50
CKM	sc-15164	Santa Cruz Biotechnology	WB	1:200
CKB	sc-15157	Santa Cruz Biotechnology	WB	1:200
mtCKs	sc-15168	Santa Cruz Biotechnology	WB	1:400
AKT	A00301	GenScript, Antibodies-online GmbH	WB	1:2000
pS-AKT	A00272	GenScript, Antibodies-online GmbH	WB	1:4000
pT-AKT	A00275	GenScript, Antibodies-online GmbH	WB	1:2000
OXPHOS	ab110412	Abcam	IF	1:200
GAPDH	Sc-25778	Santa Cruz Biotechnology	WB	1:5000
Bcl-2	SAB4500003	Sigma-Aldrich	WB	1:1000
BAX	ab7977	Abcam	WB	1:1000

Next day, the membranes were washed in TBS (3x10min) and incubated for 1 h at room temperature with appropriate anti-rabbit (GE Healthcare Amersham) or anti-goat (Santa Cruz Biotechnology) secondary antibody conjugated with horseradish peroxidase. Proteins were detected by enhanced chemiluminiscence (ECL) substrate (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific Pierce) and visualized by the LAS-4000 imaging system (Genetica, Fujifilm). Changes in the relative protein levels were quantified using the Quantity One Software (Bio-Rad). The same amount of protein (20 μg per lane) was loaded on the gels. Samples from each experimental group and from both ventricles were always run on the same gel and quantified on the same membrane. The analysis was repeated at least four times for each antibody and the results were normalized to the total protein. In the Chapter 4, GAPDH was used as a loading control to ensure that equal amount of protein was analyzed in each sample. Variation of the GAPDH immunoreactivity signal between the individual samples on each immunoblot was under 5% (*Figure 16*). In the Chapter 5 and 6, the total protein concentration was used as the most suitable referential value due to the fact that the housekeeping protein GAPDH is affected by chronic hypoxia (Balkova et al., 2011).

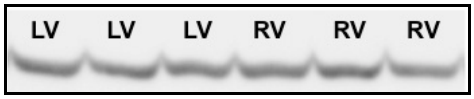

Figure 16: *The GAPDH immunoreactivity between the left (LV) and right (RV) ventricle.*

3.8 Isolation of mitochondria

Normoxic rats and rats adapted to intermittent hypobaric hypoxia were killed by decapitation. Hearts were quickly removed and washed in ice-cold saline buffer. The LV, RV, and S were separated, weighed, and immediately placed in the BIOPS buffer (10 mM Ca-EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM PCr, pH 7.1). The tissue was cut to pieces and homogenized with homogenization buffer (0.25 M sucrose, 10 mM TRIS, 2 mM EDTA, 2 mM EGTA, BSA (0.5mg/mL), pH 7.2) in the ratio 1:20 (w/v) using glass-teflon homogenizer (500-700 rpm) until the sample was homogeneous. After that, homogenates were centrifuged

at 600 g and 4°C for 10 min. The supernatants were filtered and centrifuged at 10,000 g and 4°C for 10 min. Supernatants representing the cytosol were stored at -80°C for further analyses. Pelets were resuspended in the buffer (pH 7.2) containing 0.25 M sucrose and 10 mM TRIS and centrifuged at 10,000 g and 4°C for 10 min to wash mitochondria. Supernatants were discarded and pelets were resuspended in the same buffer (0.25 M sucrose, 10 mM TRIS, pH 7.2). All contents were removed into glass homogenizer and shortly gently homogenized. Homogenates were stored at -80°C for further analyses.

3.9 Enzyme analyses

3.9.1 CK and HK activities

The specific CK and HK enzyme activities were assessed by enzyme-coupled assays and measured spectrophotometrically using a multireader system Synergy^{TM} HT (Biotek Instruments). Both assays were based upon the reduction of NAD^+ through a coupled reaction with glucose-6-phosphate dehydrogenase (G6P-DH) and were determined by measuring the increase in absorbance at 339 nm:

$$PCr + ADP \xrightarrow{CK} Cr + ATP$$

$$Glucose + ATP \xrightarrow{HK} Glucose\text{-}6\text{-}P + ADP$$

$$Glucose + NAD^+ \xrightarrow{G6P\text{-}DH} Gluconate\text{-}6\text{-}P + NADH + H^+$$

The specific enzyme activity of the HK was assessed according to a slightly modified Worthington protocol (Worthington Biochemical Corporation). The assay buffer consisted of 0.05 M TRIS, 13.3 mM $MgCl_2$, 0.8 mM NAD, 0.8 mM ATP, 0.5% Triton X-100, and 1 U/ml G-6-P dehydrogenase (pH 8.0). Samples (60 µg per well) were placed into 96-well plates. The 167 µl of the HK assay buffer were added into each sample. The reaction was initiated after 2 min by the addition of 33 µl of the starting solution (1.5 M glucose in TRIS-$MgCl_2$ buffer, pH

8.0) and the assay was run at 30°C for 15 min. The CK activity was measured at 37°C for 10 min using a commercial kit (CK NAC liq. SYS 1, Roche Diagnostics). Samples (42 µg per well) were pipetted into wells, then 200 µl of the CK working solution R1 were added and after 2 min incubation the 40 µl of CK working solution R2 were added. The specific enzyme CK and HK activities were expressed as units per gram protein (U/g) (Units of Enzyme Activity, 1979).

3.9.2 LDH release

The LDH release was determined using the LDH Liqui-UV kit (Stanbio, Boerne, TX, USA). The LDH assay was freshly prepared by mixing a solution of R1 and R2 in the ratio 5:1 (v/v). The 200 µl of the assay were added into each effluent sample (10 µl). The LDH activity was measured at 37°C, 340 nm in 96-well plates using a multireader Synergy^{TM} HT (Biotek Instruments) for 20 min and expressed as units per liter (U/L) according to manufacturer´s instructions.

3.10. Immunofluuorescence analyses

3.10.1 Native frozen section preparation

Animals were killed by decapitation. Hearts were rapidly excised, washed in ice-cold saline and placed on Langendorff apparatus, where they were perfused by Krebs-Henseleit solution with 20 mM 2,3-butanedione monoxime for 2 min. After that, atria were cut out and ventricles were cut transversally in one third from apex and snap-frozen and stored in liquid nitrogen. Frozen ventricles were mounted using tissue-Tec medium and cut to cryosections (5 to 7 µm) using a cryocut (Leica 1800). Cryosections were collected on the Super-Frost slides. Eight cryosections from each normoxic or hypoxic heart were used for antibodies staining and as negative and positive controls.

3.10.2 Formaldehyde perfused frozen section preparation

Animals were killed by decapitation. Hearts were rapidly excised, washed in ice-cold saline and perfused by Tyrode solution (140 mM NaCl, 5.4 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2.6H_2O$, 10 mM glucose, 5 mM HEPES, pH 7.4) without calcium for 2 min. Then, hearts were perfused by 4% methanol-free formaldehyde (Polysciences, Inc.) for 3 min. After that, hearts were incubated in 20% sucrose overnight. Then, atria were cut out, ventricles were cut transversally in one third from apex, snap-frozen in liquid nitrogen and stored in -80°C. The following steps were the same as in the previous procedure.

3.10.3 Immunofluorescence staining

Ventricular myocardium for immunofluorescence staining was processed as described previously (Hlavackova et al., 2010). The subcellular localization of HK1 and HK2 and their co-localization with the mitochondrial membrane in the LV and RV were investigated by immunofluorescence staining of apex cross cryosections followed by fluorescence microscopy. Cryosections were fixed in 4% methanol-free formaldehyde (Polysciences, Inc.) for 5 min at room temperature and permeabilized in 100% ice-cold methanol for 10 min. Non-specific binding sites were blocked for 1 h at room temperature in an appropriate serum diluted in PBS containing 0.3% Triton X-100 and 0.3 M glycine (Sigma-Aldrich). Cryosections were incubated for 1 h at room temperature with rabbit primary polyclonal antibodies against HK1 and HK2 from Santa Cruz Biotechnology and from Abcam (*Table 3*). Sections were subsequently incubated for 45 min at room temperature with donkey anti-rabbit IgG secondary antibody (1:200) conjugated with Alexa Fluor 488 (Invitrogen, Molecular Probes). The mitochondrial compartment was stained with MitoProfile BlueNative OXPHOS Antibody Cocktail (Abcam) overnight at 4°C and subsequently with goat anti-mouse Alexa Fluor 647 secondary antibody (1:200) for 45 min at RT (Invitrogen, Molecular Probes). Sections were mounted in ProLong Gold Antifade Reagent containing a nuclei marker 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Molecular Probes) and stored at 4°C.

3.10.4 Quantitative fluorescence microscopy

Representative qualitative images were captured by confocal microscopy (Leica TCS SP2). Images were acquired using a sequential scanning mode avoiding cross-talk between channels, 16 times line averaging, and noise reduction by 3 times frame averaging. For the purpose of quantitative analyses, images were acquired using a widefiled fluorescence microscope (Olympus Cell^R IX2-UCB). The objective lens magnifications were 20x 0.75 NA Plan-Apochromat (Chapter 4) and 100x 1.4 NA Plan-Apochromat (Chapter 5 and Chapter 6). The excitation and emission spectra corresponded to used fluochromes: DAPI (345/455), Alexa 488 (495/519), Alexa 647 (650/668). Images were acquired using Hamamatsu ORCA camera C4742-80-12AG and the fluorescence intensity was measured using Fiji ImageJ open source software (Schindelin et al., 2012). For the quantification of fluorescence intensity (Chapter 4), eight positions (four for each ventricle) on every section were captured. A time-lapse scanning mode was used for in order to prove the stability of fluorescence during the sample observation and acquisition. Each position was scanned 10 times and average intensity for each ventricle was calculated. For the co-localization screenings, eight positions on each cryosection (four for each ventricle) were sequentially acquired for red (AlexaFluor 647) and green (AlexaFluor 488) channels. Each position was optically sectioned at 0.5 μm steps resulting in approximately 12 focal planes depending on specimen thickness. Each position was captured twice to calculate the influence of the noise of an image. Regions of interest (ROI) were selected as sarcoplasmic myofibrilar regions excluding nucleus, perinuclear area and sarcolemma. The correlation between the fluorescence signal of HK1 or HK2 isoforms and the mitochondrial OXPHOS complex was calculated using an ICA plugin of Fiji ImageJ software (Li et al., 2004). The RBNCC method (Adler et al., 2008) was applied for the correction of noise when calculating the Pearson's correlation coefficient between the green channel representing HK1 or HK2 and the red channel representing OXPHOS complexes.

3.11 Statistical analyses

3.11.1 Study 1: The comparison of the LV and RV under normoxia

Ten rat hearts were used for WB, enzyme activities, and Real-Time RT-PCR analyses and 6 hearts were used for quantitative fluorescence analyses. The statistical differences between the ventricles were determined by the unpaired Mann Whitney test ($P < 0.05$ or $P < 0.001$) (Chapter 4).

3.11.2 Study 2: The effect of the normobaric hypoxia and I/R insult on the CK and HK enzymes

Five hearts from each experimental group were used for WB, enzyme activities, and Real-Time RT-PCR analyses and 6 hearts were used for quantitative fluorescence analyses and I/R protocol. Statistical evaluation was performed using One-way ANOVA followed by a *post hoc* Dunett's multiple comparison test, One-way ANOVA followed by a *post hoc* Tukey's multiple comparison test, and Mann Whitney test. Values of $P < 0.05$ were considered statistically significant (Chapter 5).

3.11.3 Study 3: The effect of the hypobaric hypoxia on the CK and HK enzymes

Five hearts from each experimental group were used for WB, enzyme activities, and Real-Time RT-PCR analyses and 6 hearts were used for quantitative fluorescence analyses. The statistical differences between normoxic and hypoxic groups were determined by the unpaired Mann Whitney test ($P < 0.05$) (Chapter 6).

All statistical analyses were performed using GraphPad Prism 5.00 software. All data are expressed as mean ± S.E.M. The degree of imunoreactivity, relative mRNA expression, and fluorescence intensity are expressed as a percentage of total. The co-localization of HK with mitochondria is expressed as the Pearson's correlation coefficient and enzyme activities are expressed as U/g or U/L.

4. STUDY 1: The comparison of the LV and RV under normoxia

4.1 Introduction

The normoxic RV and LV differ in many anatomical and physiological parameters (reviewed in Haddad et al., 2008; Walker and Buttrick, 2009). The ventricular pressure and elasticity of the RV is lower than that of the LV (Davidson and Bonow, 2005; Dell'Italia and Walsh, 1988; Starling et al., 1987). The RV free wall is thinner than the LV free wall (Ho and Nihoyannopoulos, 2006; Jiang, 1994) and the mass of the RV is also less that of the LV (Lorenz et al., 1999). While the LV has an ellipsoidal shape, the RV is crescent shaped in cross-sections and appears triangular from the side view (Dell'Italia, 1991; Ho and Nihoyannopoulos, 2006; Jiang, 1994). It has been reported that there are regional differences in the size of cardiomyocytes in adult rat hearts (Gerdes et al., 1985), and that cardiomyocytes respond differently to changes in hemodynamic load depending on the ventricle in which they are located (Campbell et al., 1991). Concerning myocardial energetics, oxygen requirements of the RV is lower compared to the LV due to lower wall stress, resulting in larger oxygen supply reserve (Walker and Buttrick, 2009) making the RV less vulnerable to conditions associated with increased energy demands. Based on these differences, LV and RV could respond diversely to various physiological conditions, such as physical training, high altitude exposure or pregnancy, as well as to pathophysiological situations, such as systemic and pulmonary hypertension, sleep apnea and others (reviewed in Walker and Buttrick, 2009). Some physiological studies have demonstrated that the RV is more resistant than the LV to toxic effect of anthracycline and to injury caused by acute ischemia (Baker et al., 1999; Belham et al., 2006; Dell'Italia, 1991), which may suggest the existence of some specific protective mechanism(s) operating in the RV.

As mentioned previously, CK and HK play an essential role in energy homeostasis and possess an anti-apoptotic effect (Chapter 1.2 and 1.3). In addition, AKT enhances the pro-survival effect of HK. Therefore, the purpose of the present study was to compare the expression and subcellular localization of HK isoforms, AKT kinase, and CK isoforms between ventricles of male Wistar rats. The similar comparison study at the protein and mRNA levels has not yet been examined.

4.2 Results

The Real-Time qRT-PCR analyses showed a significantly higher mRNA levels of CK isoforms in the RV as compared to the LV (*Figure 17*), however the protein levels of all CK isoforms (*Figure 17*) as well as the total CK activity (*Figure 18*) remained unchanged.

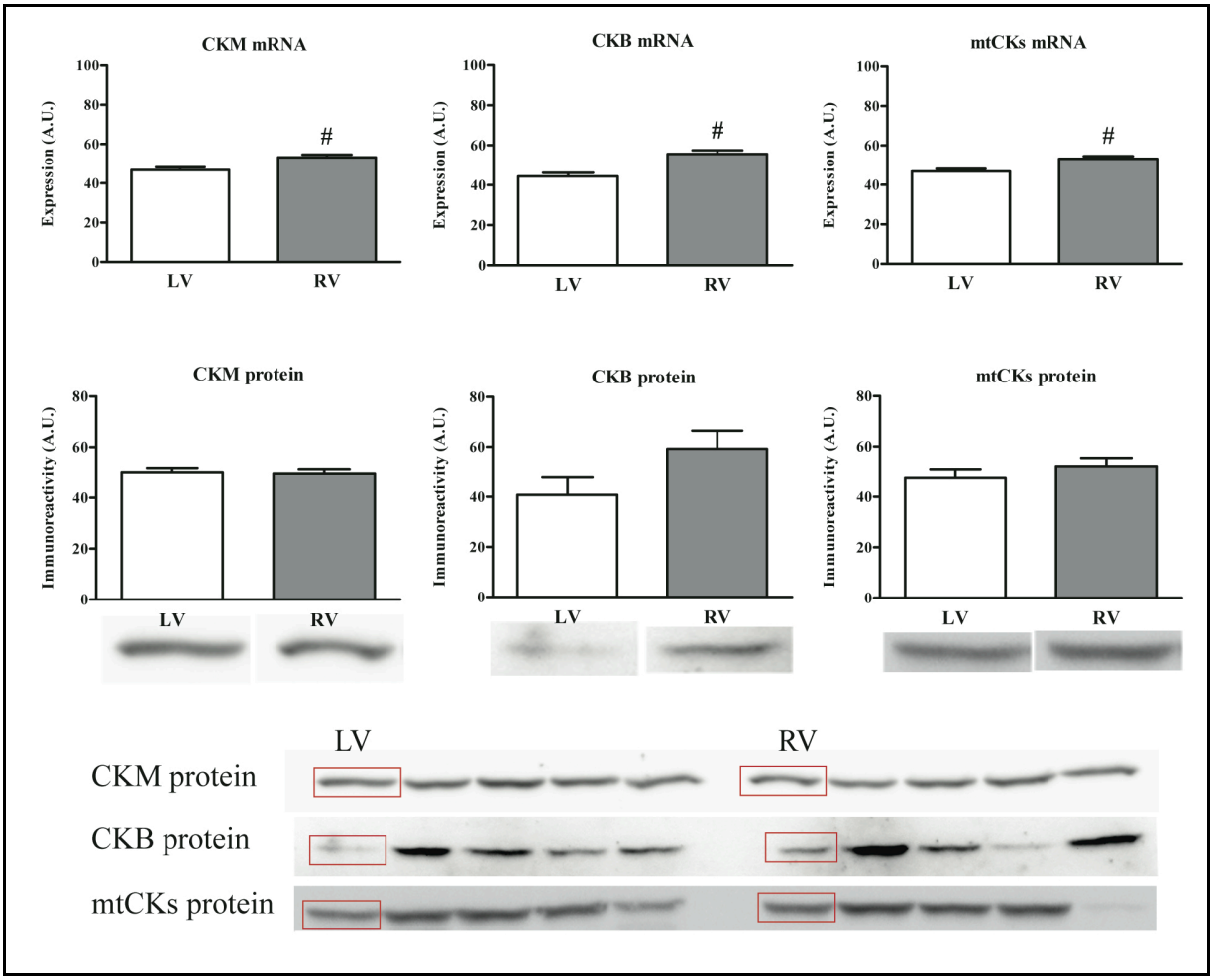

Figure 17: *The expression of CK isoforms in the normoxic left (LV) and right (RV) ventricles. The relative levels of the CKM mRNA (top left), CKB mRNA (top middle), mtCKs mRNA (top right), CKM protein (middle left), CKB (middle middle), and mtCKs protein (middle right) are expressed as a percentage of total. The representative bands were cut out from the original gels containing also other experimental groups (bottom). Values are mean ± S.E.M. (n = 5). # P < 0.05 vs. LV.*

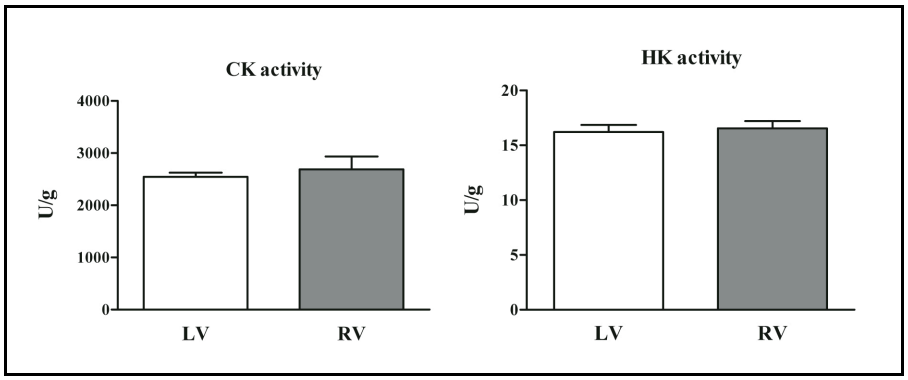

Figure 18: *The specific enzyme activities of the CK (left) and HK (right) in the normoxic left (LV) and right (RV) ventricles. The CK and HK activities are expressed as units per gram protein (U/g). Values are mean ± S.E.M. (n = 5).*

The mRNA levels of both HK1 and HK2 were significantly higher by ~ 128% and 34%, respectively, in the RV than in the LV (*Figure 19*). The protein levels of the HK1 and HK2 followed this pattern: they were also markedly higher (by ~ 76% and 42%, respectively) in the RV than in the LV (*Figure 19*). The HK specific enzyme activity did not significantly differ between the ventricles (*Figure 18*).

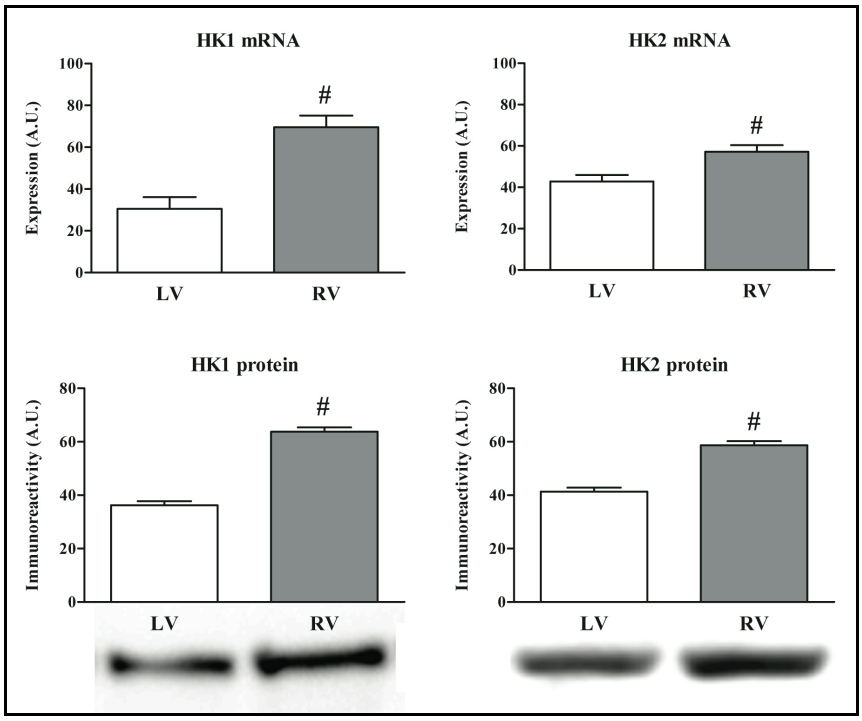

Figure 19: *The expression of HK1 and HK2 isoforms in the normoxic left (LV) and right (RV) ventricles. The relative levels of the HK1 mRNA (top left), HK2 mRNA (top right), HK1 protein (bottom left), and HK2 protein (bottom right) are expressed as a percentage of total. Values are mean ± S.E.M. (n = 10). # P < 0.05 vs. LV.*

The expression of both HK1 and HK2 isoforms in the RV was also verified by the immunofluorescence staining of the cross cryosections. The HK1 as well as HK2 manifested the significantly higher fluorescence intensity (by ~ 24% and 22%, respectively) in the RV compared to the LV (*Figure 20*).

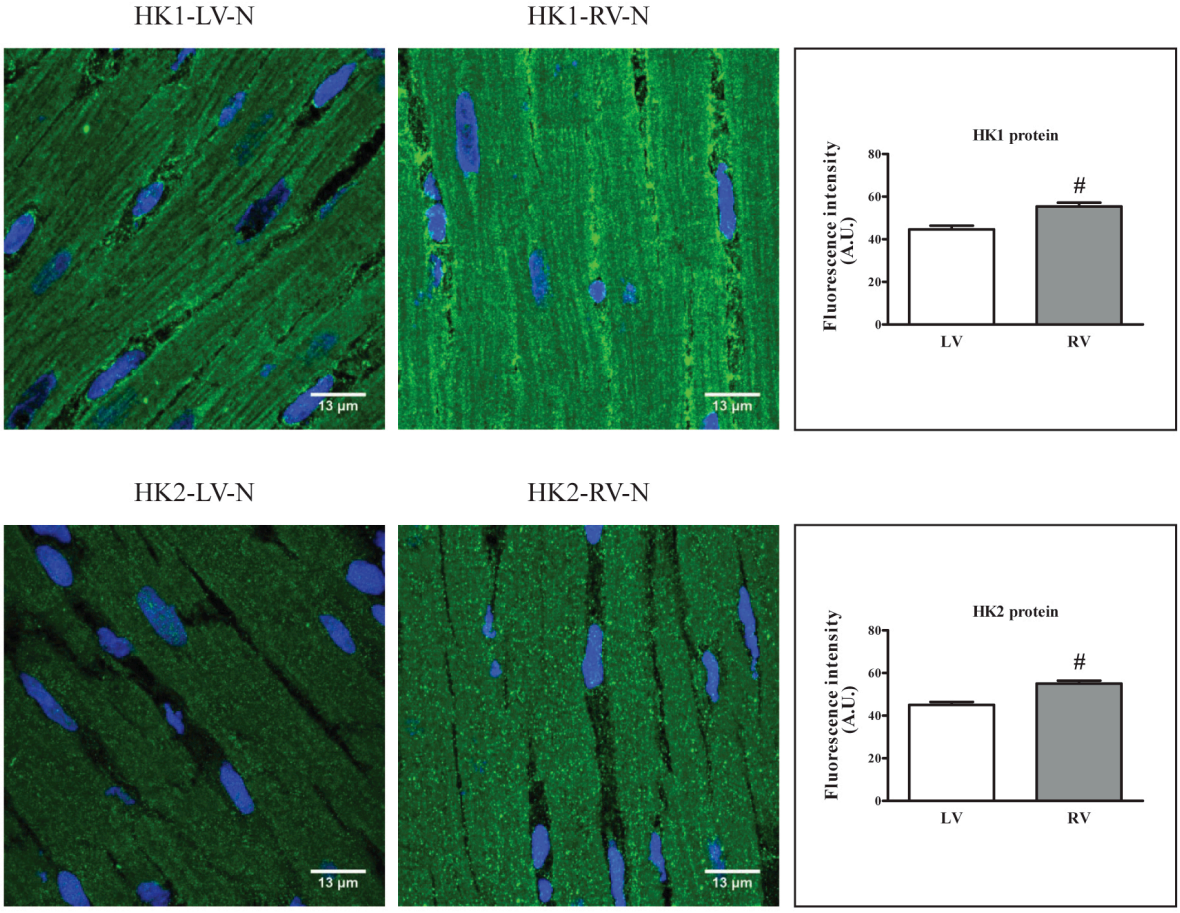

Figure 20: *The determination of the intensity of HK1 and HK2 fluorescence signals in the left (LV) and right (RV) ventricles of normoxic rat hearts. The representative micrographs show the intensity of fluorescence signals of HK1 in the LV (top left), HK1 in the RV (top middle), HK2 in the LV (bottom left), and HK2 in the LV (bottom middle). The green color corresponds to the specific HK1 and HK2 staining and the blue color indicates the nuclear 4',6-diamidino-2-phenylindole (DAPI) staining. The scale bar represents 13 µm. The quantification of the intensity of HK1 (top right) and HK2 (bottom right) fluorescence signals are expressed as a percentage of the total. Values are mean ± S.E.M. (n = 6). # P < 0.001 vs. LV.*

The phospho-Ser473-AKT as well as the phospho-Thr308-AKT level was significantly higher in the RV than in the LV (*Figure 21*), similarly as the ratio of the phosphorylated to non-phosphorylated AKT (*Figure 21*), indicating a higher activation of the AKT in the RV, which is required for the interaction of HK2 with mitochondria.

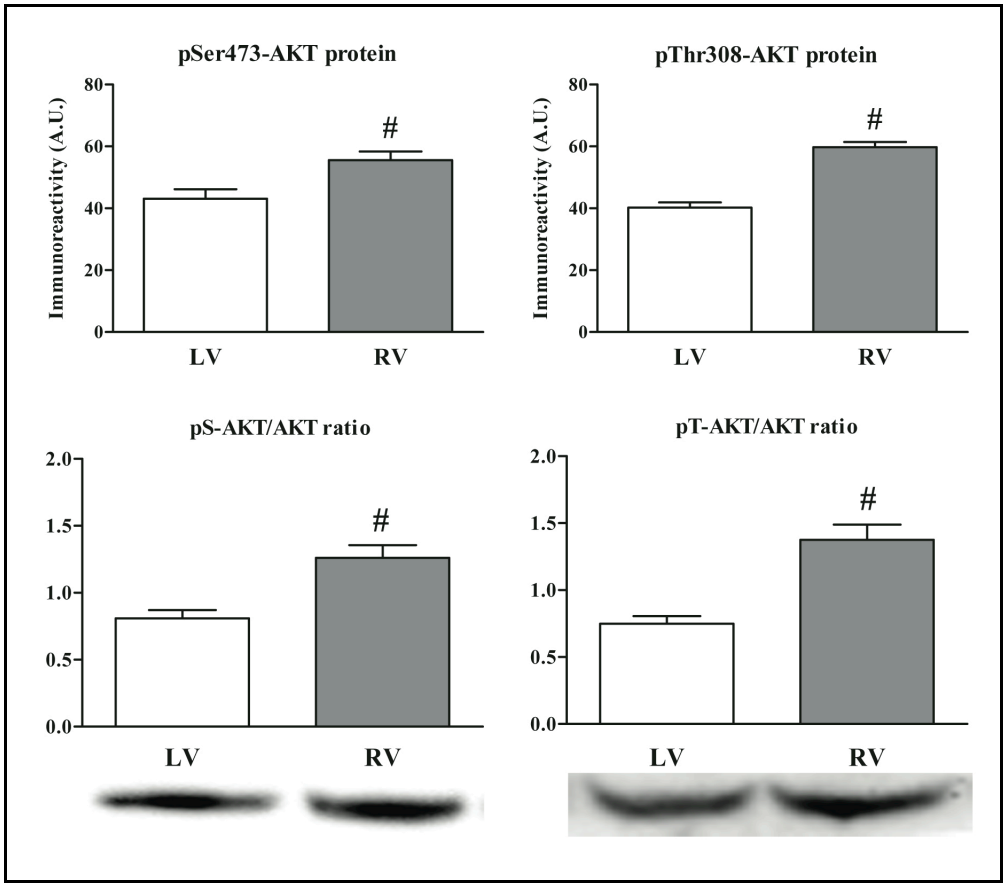

Figure 21: *The expression of the phosporylated AKT at Ser473 residue (pS-AKT) and Thr308 residue (pT-AKT) and the determination of the ratio of the phosphorylated/non-phopshorylated AKT: pS-AKT/AKT (bottom left) and pT-AKT/AKT (bottom right) in the normoxic left (LV) and right (RV) ventricles. The relative protein levels of the pS-AKT (top left) and pT-AKT (top right) are expressed as a percentage of total. Values are mean ± S.E.M. (n = 10). # P < 0.05 vs. LV.*

It has been previously demonstrated that the higher phosphorylation of the AKT is usually associated with a higher co-localization of HK2 with mitochondria (Miyamoto et al., 2008, Roberts et al., 2013). As can be seen in *Figure 20*, the HK isoforms displayed different patterns of fluorescence signals in cardiomyocytes. In contrast to a fuzzy pattern of the HK2 distribution, the HK1 appeared to be organized rather into longitudinal arrays or chains. The Pearson's correlation coefficients between the green (HK1 or HK2) and the red channels (OXPHOS mitochondria) were calculated to further quantify differences in the co-localization of HKs with mitochondria. Neither co-localization of the HK1 (0.48 ± 0.04 *vs.* 0.53 ± 0.04) nor co-localization of the HK2 (0.35 ± 0.03 *vs.* 0.37 ± 0.03) with mitochondria differed

between the LV and the RV. However, the co-localization of the HK2 with mitochondria was significantly lower compared to the HK1 in both ventricles (*Figure 22*).

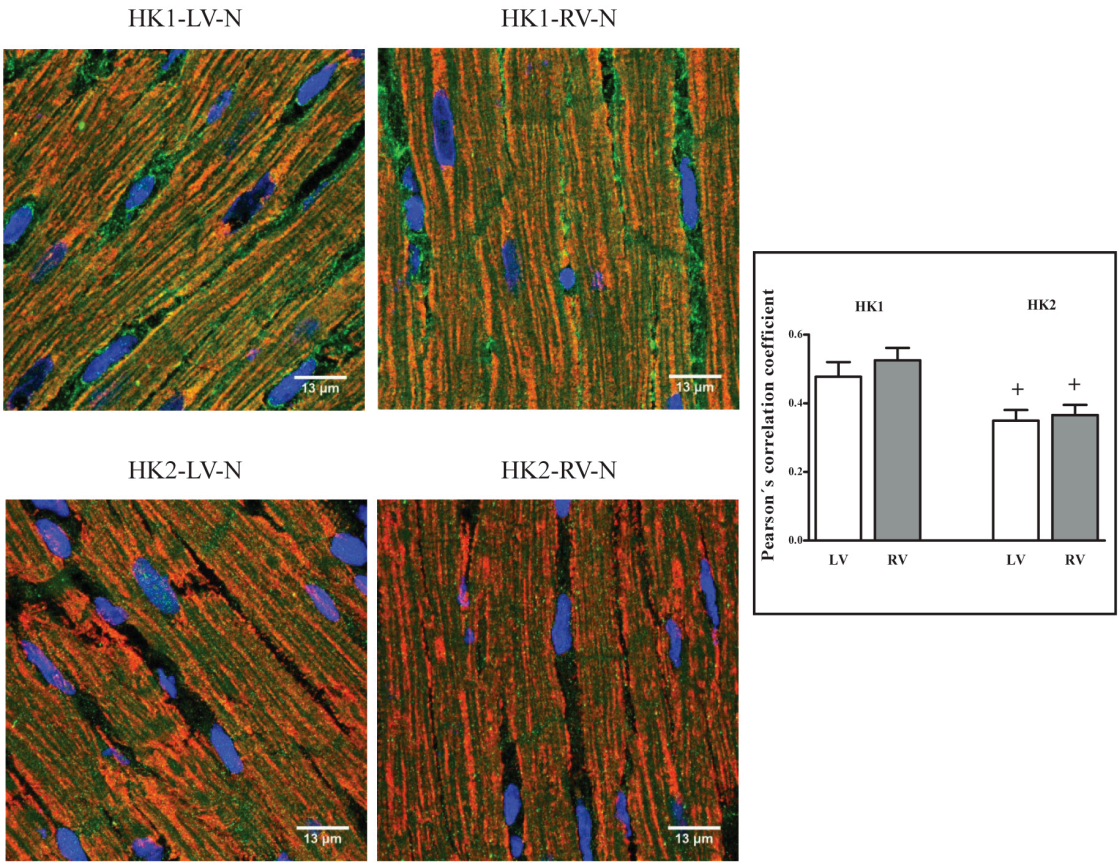

Figure 22: *The representative micrographs showing the co-localization of the HK1 (top left and top middle) and HK2 (bottom left and bottom middle) with mitochondria in cross cryosections of the normoxic left (LV) and right (RV) ventricles obtained by a confocal microscope. The green color represents the specific HK1 or HK2 staining, the blue color indicates the nuclear 4',6-diamidino-2-phenylindole (DAPI) staining, and the red color represents the distribution of the OXPHOS complexes. The increase in yellow-orange color in both panels indicates an increased co-localization of the HK1 or HK2 with mitochondria in both LV and RV. The scale bar represents 13 µm. The HK1 and HK2 co-localizations with mitochondria (OXPHOS complexes) were quantified using the Pearson´s correlation coefficients (middle right). Values are mean ± S.E.M. (n=6). +P < 0.05 vs. HK1.*

4.3 Conclusion

The present study has revealed significantly higher amounts of the HK1 and HK2 and the increased phosphorylation of the AKT in the RV as compared to the LV. These findings suggest that the AKT activation is a necessary but not a sufficient condition for the enhancement of the interaction of the HK2 with mitochondria and that yet another mechanism may exist in the RV. In conclusion, these results suggest that the RV has a higher activity of aerobic glycolytic metabolism and may be able to respond faster and more powerfully to stressful stimuli than the LV.

5. STUDY 2: The effect of the normobaric hypoxia and I/R insult on the CK and HK enzymes

5.1 Introduction

The adaptation to the normobaric hypoxia has been reported to impair cardiac energetics in humans by decreasing the PCr/ATP ratio (Holloway et al., 2011), which could reflect the participation of other phosphotransfer systems in the stimulation of the ATP production (discussed in details in Dzeja and Terzic, 2003; Dzeja et al., 2011). However, another study has shown that the adaptation to the normobaric hypoxia significantly increases the PCr synthesis in rat hearts (Novel-Chate et al., 1995), which could indicate a higher activity of the mtCK. The normobaric hypoxia has not been studied as much as the hypobaric hypoxia. So far, only one paper has been published for the CK. Novel-Chaté et al. (1998) have reported a significant decrease of the total CK and mtCKs isoform enzyme activities in the LV of rats adapted for 3 weeks to the normobaric hypoxia (10% O_2). The CKM isoform activity also decreased but not significantly, and the CKB activity remained unchanged (Novel-Chate et al., 1998). The adaptation to a chronic hypoxia did not affect either the CK activity or alterations in the CK isoform distribution in the RV. This study, dealing with the CK under hypoxia, have focused only on the enzyme activity of the total CK or each CK isoform. The expression on the mRNA as well as protein level has not yet been investigated. The similarly focused papers have been also published with the HK. Rumsey et al. (1999) have reported an increase of the HK activity and mRNA level in the LV as well as in the RV of rats adapted to the normobaric hypoxia (10% O_2) for 14 days and 21 days, respectively (Rumsey et al., 1999). Daneshrad et al. (2000) have also showed an increased HK activity in the LV and in the RV of rats adapted to the normobaric hypoxia (10% O_2) for 3 weeks (Daneshrad et al., 2000). However, it is unknown whether the continuous and intermittent normobaric hypoxias have a different impact on the CK and HK activities and isoform profiles. It has been shown that the continuous normobaric hypoxia (CNH) as well as the intermittent hypoxia lasting 8 h per day (INH-8) increased the cardiac tolerance to the ischemic injury, while the regimen based on 23 h of hypoxia per day interrupted with only 1 h normoxic episode (INH-23) did not induce the cardioprotective phenotype (*Figure 23*) (Kasparova et al., data in preparation; Neckar et al.,

2013). Therefore, the purpose of the present study was to compare the protective and non-protective regimens and to determine the CK and HK responses to the I/R insult in the LV of rats adapted to protective CNH regimen.

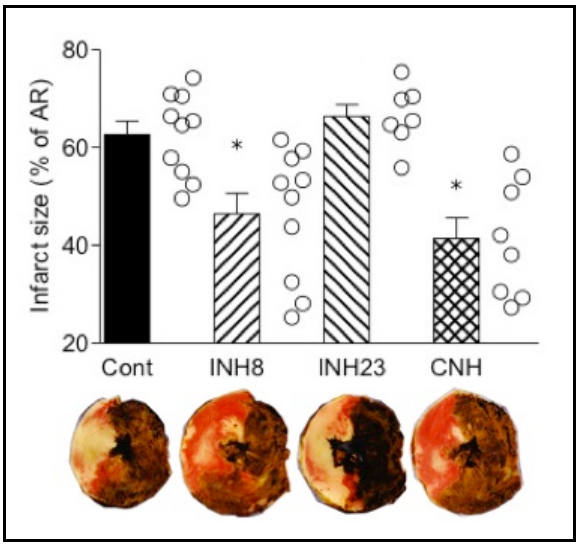

Figure 23: *The comparision of protective and non-protective regimens of normobaric hypoxia. The Cont, normoxic controls; CNH, continuous hypoxia; INH-23, intermittent hypoxia for 23 h/day; INH-8, intermittent hypoxia for 8 h/day. The infarct size is expressed as a percentage of area at risk (AR) and is shown at representative images below the bar chart: brown color represents normally perfused tissue stained by potassium permanganate; red area, tetrazolium positive, represents tissue surviving the occlusion; and pale area, tetrazolium negative, is infarcted tissue. The figure is adapted from Dr. Neckar with permission.*

5.2 Results

5.2.1 The effect of the normobaric hypoxia

As shown in the *Table 1* (Chapter 3.5), the adaptation of rats to the INH-23 and CNH regimens led to a growth retardation by ~ 14% as compared to normoxic controls. The INH-8 regimen had no significant effect on the body weight. While the hypoxia did not significantly affect the LV weight, it resulted in the RV hypertrophy, which was more pronounced in the CNH and INH-23 groups than in the INH-8 group. Similarly, the increase of the hematocrit was proportional to the duration of daily hypoxic exposure.

The adaptation to all hypoxic regimens affected neither mRNA nor protein levels of the CKM in both ventricles, except for a slight decrease of the LV transcript in the INH-23 group (*Figure 24*). On the other hand, the normobaric hypoxia up-regulated the CKB at the mRNA and protein levels in both LV and RV, although only some groups exhibited a statistically significant difference from normoxic controls due to a rather high variability in this isoform level (*Figure 24*). The protein expression of the mtCKs was significantly increased in the LV after the adaptation to all hypoxic regimens (by ~ 33%, 29%, and 30% in the CNH, INH-23, and INH-8 groups, respectively), while the LV levels of the mtCKs mRNA increased only in the CNH and INH-8 groups. In contrast, no effect of the hypoxia on the mtCKs expression was observed in the RV (*Figure 24*).

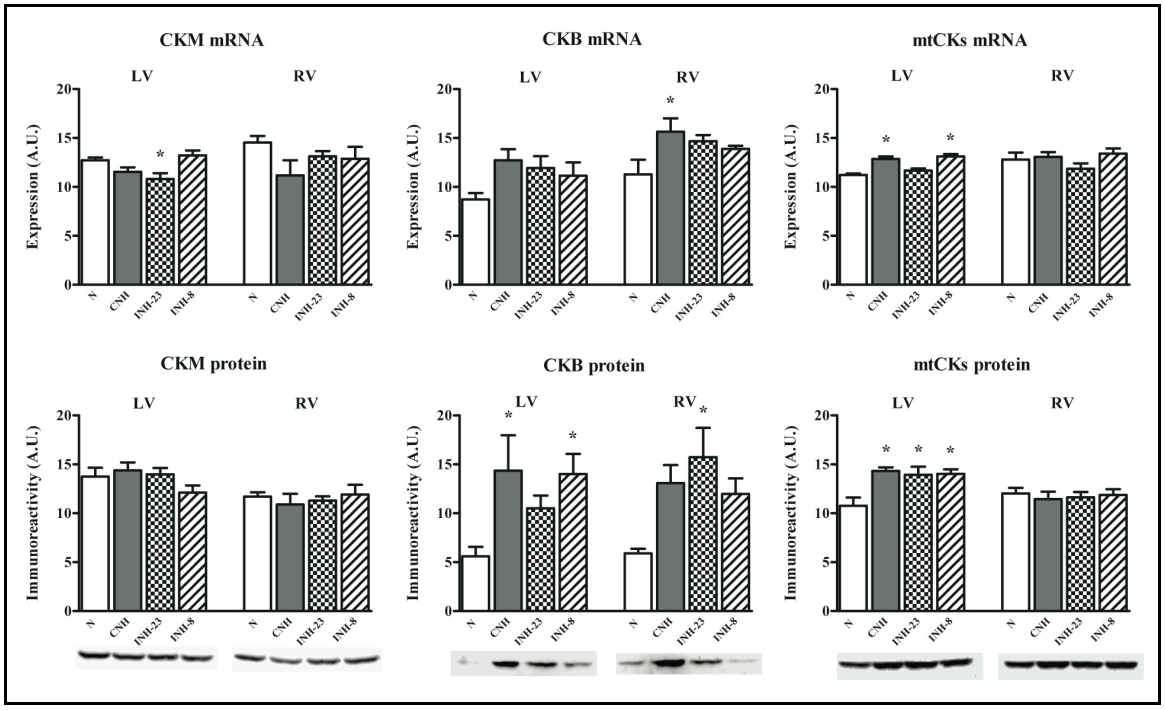

Figure 24: *The expression of CK isoforms in the left (LV) and right (RV) ventricles. The relative levels of the CKM mRNA (top left), CKB mRNA (top middle), mtCKs mRNA (top right), CKM protein (bottom left), CKB protein (bottom middle), and mtCKs protein (bottom right) are expressed as a percentage of total amount determined in the LV and RV from normoxic rats (N), from rats adapted to the continuous normobaric hypoxia (CNH), intermittent normobaric hypoxia for 23 h/day (INH-23), and intermittent normobaric hypoxia for 8 h/day (INH-8). Values are mean ± S.E.M. (n = 5). * P < 0.05 vs. N.*

The protein and mRNA levels of both HK isoforms were markedly higher in the normoxic RV than in the LV, which is in line with the previous findings (Chapter 4.2). All hypoxic regimens similarly increased the HK1 protein levels in the LV (by ~ 99%, 95%, and 86% in the CNH, INH-23, and INH-8 groups, respectively), but did not affect the HK1 mRNA levels. Interestingly, the adaptation to a moderate normobaric hypoxia affected neither mRNA nor protein levels of the HK1 in the RV (*Figure 25*). All regimens of the hypoxia up-regulated both mRNA (by ~ 74-112 %) and protein (by ~ 94-113 %) level of the HK2 in the LV. In the RV, the protein expression of the HK2 remained unchanged and the mRNA level increased only in the INH-8 group (*Figure 25*).

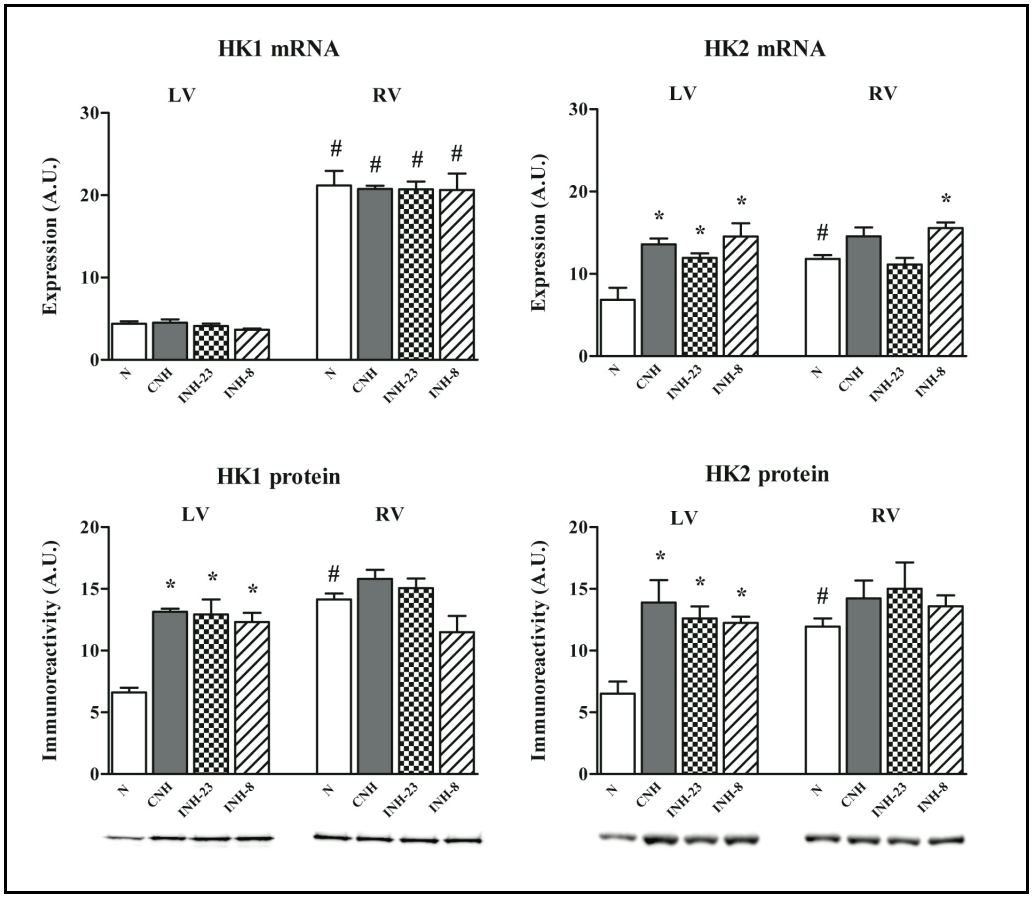

Figure 25: *The expression of HK1 and HK2 isoforms in the left (LV) and right (RV) ventricles. The relative levels of the HK1 mRNA (top left), HK2 mRNA (top right), HK1 protein (bottom left), and HK2 protein (bottom right) are expressed as a percentage of total amount determined in the LV and RV from normoxic rats (N), from rats adapted to the continuous normobaric hypoxia (CNH), intermittent normobaric hypoxia for 23 h/day (INH-23), and intermittent normobaric hypoxia for 8 h/day (INH-8). Values are mean ± S.E.M. (n = 5). * P < 0.05 vs. N; # P < 0.05 vs. corresponding LV.*

The adaptation to a moderate normobaric hypoxia markedly increased the total CK activity in the LV by ~ 41% (CNH), 43% (INH-23), and 40% (INH-8), which was in agreement with the enhanced expression of the mtCKs and CKB proteins. In the RV, only adaptations to the INH-23 and INH-8 regimens significantly increased the total CK activity by ~ 31% and 51%, respectively (*Figure 26*). All hypoxic regimens also significantly increased the HK activity in the LV by ~ 32% (CNH), 23% (INH-23), and 29% (INH-8), which was in line with the up-regulation of the HK1 and HK2 protein levels. The total HK activity in the RV was higher than in the LV already under normoxic conditions and the normobaric hypoxia resulted in further increases by ~ 67% (CNH), 75% (INH-23), and 21% (INH-8) (*Figure 26*).

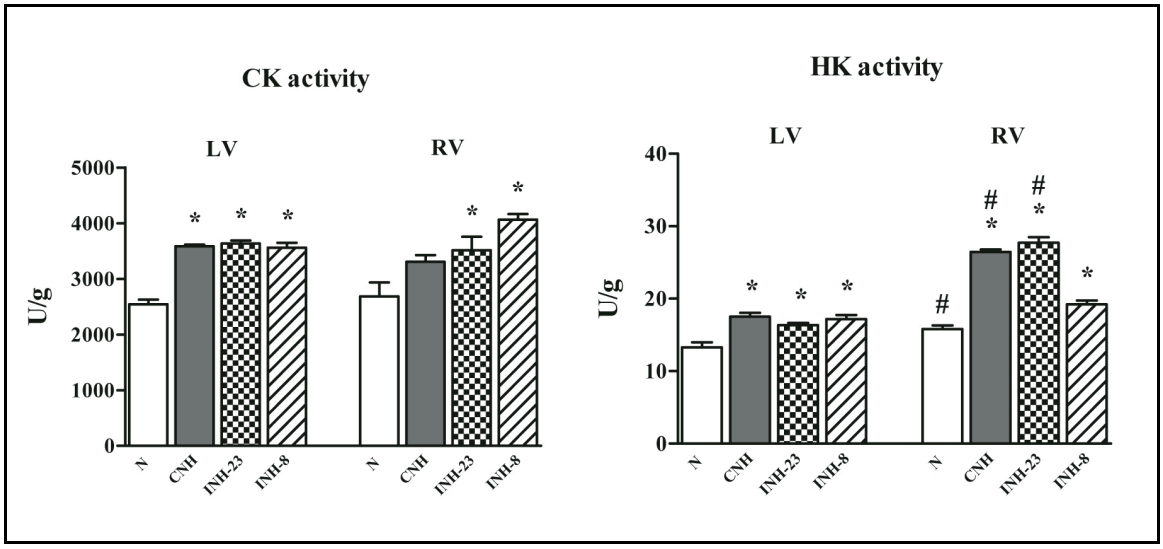

Figure 26: *The specific enzyme activities of the CK and HK in the left (LV) and right (RV) ventricles. The CK (left) and HK (right) activities are expressed as units per gram protein (U/g) in the LV and RV from normoxic rats (N), from rats adapted to the continuous normobaric hypoxia (CNH), intermittent normobaric hypoxia for 23 h/day (INH-23), and intermittent normobaric hypoxia for 8 h/day (INH-8). Values are mean ± S.E.M. (n = 5). * P < 0.05 vs. N; # P < 0.05 vs. corresponding LV.*

The fluorescence analyses showed a higher co-localization of the HK1 with mitochondria than the HK2 in the normoxic LV and RV, which is in accordance with the previous results (Chapter 4.2). In addition, a higher co-localization of the HK1 with mitochondria, compared to the HK2, was also observed in the RV of the CNH rats. However, the adaptation to the CNH did not affect the mitochondrial co-localization of both isoforms in the LV as well as in the RV, compared to the normoxic controls (*Figure 27*).

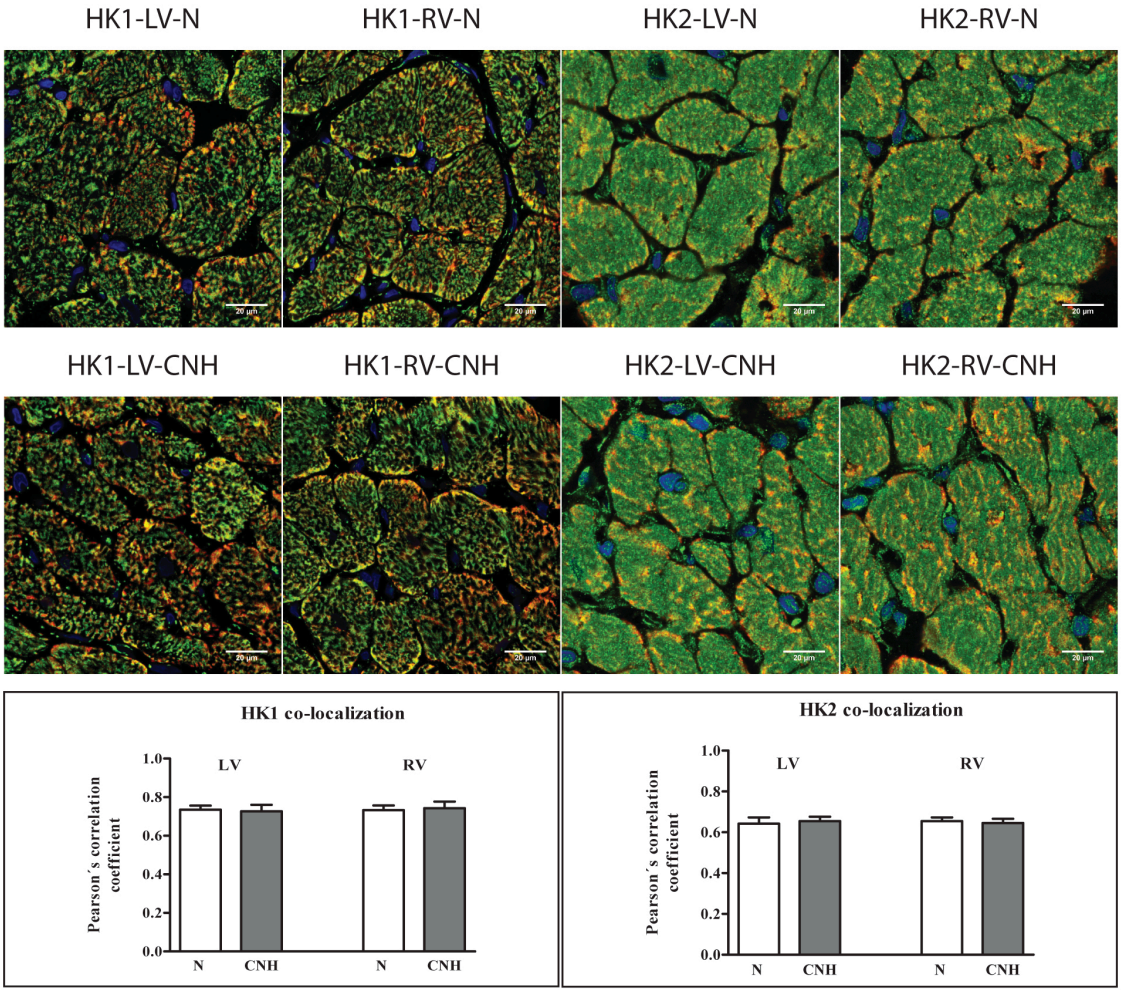

Figure 27: *The representative qualitative images of transversal cross cryosections showing the co-localization of the HK1 and HK2 with mitochondria in the left (LV) and right (RV) ventricles from normoxic rats (N) and from rats adapted to the continuous normobaric hypoxia (CNH) (top and middle). The green color represents the specific HK1 or HK2 staining and the blue color indicates the nuclear 4',6-diamidino-2-phenylindole (DAPI) staining. The red color represents the distribution of the mitochondrial compartments (I-V complexes). The increase in yellow-orange color indicates an increased co-localization of the HK1 and HK2 with mitochondria expressed as the Pearson´s correlation coefficients (bottom). The scale bar represents 20 μm. Values are mean ± S.E.M. (n = 5).*

5.2.2 The effect of the I/R insult

The present I/R protocol was designed to be marginal for the fatal cell damage. The degree of cell damage was determined by measuring the LDH release, which occurred only after 30 s and 60 s of reperfusion. Nevertheless, these increases were negligible due to low levels of the LDH activity (*Figure 28*). The degree of apoptosis was determined by the Bcl-2/BAX ratio, which remained unchanged during ischemia and I/R insult in normoxic LV. The ratio of the anti-apoptotic Bcl-2 protein to the pro-apoptotic BAX protein was significantly elevated after the I/R insult in the CNH LV, as compared to the normoxic LV (*Figure 28*).

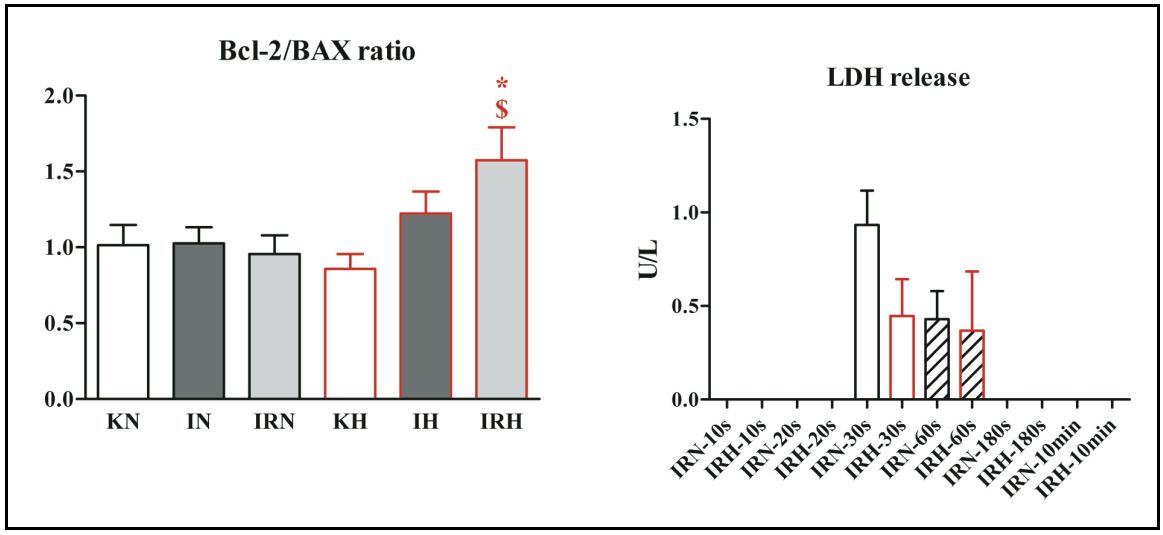

Figure 28: *The degree of cell damage and apoptosis. The ratio of the Bcl-2/BAX proteins in the left ventricle (LV) of perfused control (KN, KH), ischemic (IN, IH), and I/R (IRN, IRH) hearts. The N, normoxic rats; H, rats adapted to the continuous normobaric hypoxia (left). The LDH release during the reperfusion is expressed as U/L (right). Values are mean ± S.E.M. (n = 6).* **$** *P < 0.05 vs. KH;* ***** *P < 0.05 vs. corresponding normoxic group.*

The I/R insult did not affect the HK2 protein level in the hypoxic LV. Only ischemia had a tendency to increase the HK2 protein level in the hypoxic LV. The HK1 expression remained unchanged in all experimental groups (*Figure 29*).

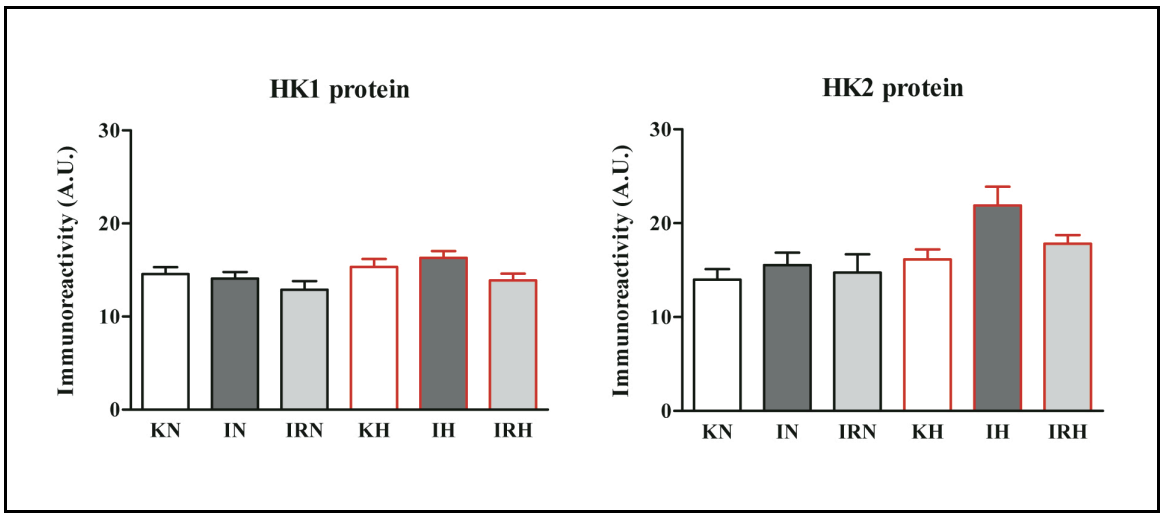

Figure 29: *The expression of the HK1 protein (left) and HK2 protein (right) in the left ventricle (LV) of perfused control (KN, KH), ischemic (IN, IH), and I/R (IRN, IRH) hearts. The N, normoxic rats; H, rats adapted to the continuous normobaric hypoxia. Values are mean ± S.E.M. (n = 6).*

The I/R insult significantly increased the total HK activity in the CNH LV compared to the appropriate normoxic LV. The ischemia also markedly elevated the total HK activity in the hypoxic LV as well as in the control LV (*Figure 30*). The total CK activity was not affected either by ischemia or by the I/R insult (*Figure 30*).

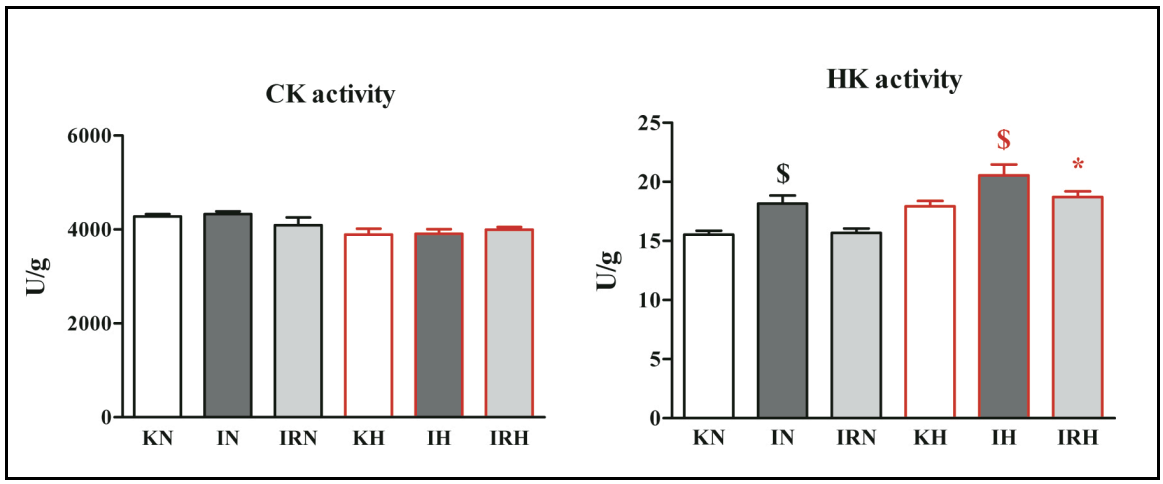

Figure 30: *The specific enzyme activities of the CK and HK in the left ventricle (LV) of perfused control (KN, KH), ischemic (IN, IH), and I/R (IRN, IRH) hearts. The N, normoxic rats; H, rats adapted to the continuous normobaric hypoxia. Values are mean ± S.E.M. (n = 6).* ***$*** *P < 0.05 vs. KN;* ***$*** *P < 0.05 vs. KH;* ******* *P < 0.05 vs. corresponding normoxic group.*

5.3 Conclusion

This study demonstrated similar effects of three different hypoxic regimens on CK and HK isoforms in the myocardium of adult rats. The up-regulation of the mitochondrial CK and HK and their activities may lead to a higher stimulation of the respiratory chain *via* ADP recycling, which can reduce a formation of ROS and thus help to prevent the oxidative stress and maintain the energy homeostasis during the normobaric hypoxia. Although any difference between the protective and non-protective phenotype was found, it cannot be ruled out that the CK and HK may play a role in the cardioprotective mechanisms induced by the adaptation to the normobaric hypoxia. The fluorescence analyses revealed that the CNH regimen stabilizes the HK bond with mitochondria in both ventricles, which can subsequently protect the HK dissociation from mitochondria under the I/R injury. This suggestion is supported by an increased HK activity in the CNH LV subjected to the I/R insult.

6. STUDY 3: The effect of the hypobaric hypoxia on the CK and HK enzymes

6.1 Introduction

The hypobaric hypoxia has been studied since 1950th. The first experiments has been carried out in the Czech Republic in 1958 by Kopecky and Daum, who have demonstrated the cardiprotective effect of the adaptation to the hypobaric hypoxia. However, the hypobaric hypoxia differ in some physiological parameters from the normobaric hypoxia (Millet et al., 2012; Savourey et al., 2003). Surprisingly, few studies focused on the CK and HK activities under hypoxic conditions have been published untill now. Pissarek et al. (1997) adapted rats to the hypobaric hypoxia for 28 days simulating altitude 5500 m. They have observed a significant decrease of the total CK activity in the LV and a non-significant decrease of the total CK activity in the RV. The enzyme activities of CK isoforms were also changed under hypoxic conditions. The CKM activity significantly decreased, while the CKB activity significantly increased in both ventricles. The mtCKs activity significantly decreased only in the LV (Pissarek et al., 1997). However, more recent research has shown different results. Letout et al. (2005) have found that the total CK activity increased in the LV but remained unchanged in the RV of rats adapted to the hypobaric hypoxia (640 hPa, 3700 m) for 3 weeks. The CKM isoform activity increased in the LV but decreased significantly in the RV. The CKB activity markedly increased in both ventricles. The mtCKs had a tendency to grow in the LV as well in the RV (Letout et al., 2005). These different findings may be due to a distinct model of the hypobaric hypoxia. According to many models of the hypobaric hypoxia, it is very complicated to compare obtained results with published data.

Only one paper has been published regarding hexokinase thus far. Cai et al. (2010) have demonstrated a decrease of the HK activity in the RV and no changes in the HK activity in the LV of rats adapted for 5 weeks to a hypobaric hypoxia (53 kPa, 5000 m) (Cai et al., 2010). The present study used a model of the hypobaric hypoxia simulating a high altitude of 7000 m, which represents greater oxidative stress and thus may have a different effect on the HK and CK functions.

6.2 Results

The adaptation to the IHH-8 substantially increased the total CK activity in both ventricles (*Figure 31*), which was in agreement with the enhanced expression of mtCKs and CKB proteins (*Figure 32*). The adaptation to the IHH-8 regimen also significantly increased the total HK activity in both ventricles (*Figure 31*), which was in line with the up-regulation of the HK1 and mainly with the HK2 protein level (*Figure 32*).

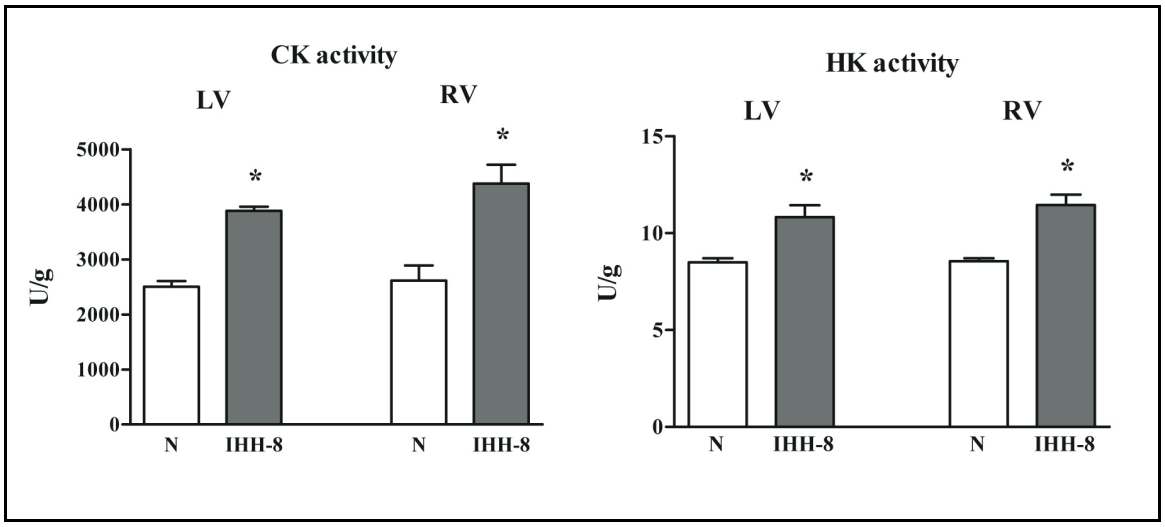

Figure 31: *The specific enzyme activities of the CK and HK in the left (LV) and right (RV) ventricles. The CK (left) and HK (right) activities are expressed as units per gram protein (U/g) in the LV and RV from normoxic rats (N) and from rats adapted to the intermittent hypobaric hypoxia (IHH-8). Values are mean ± S.E.M. (n = 5). * P < 0.05 vs. N.*

The adaptation to the severe intermittent hypobaric hypoxia significantly decreased the CKM mRNA in both ventricles and the protein level in the RV. The CKM protein in the LV only showed a tendency to decrease. On the other hand, the IHH-8 adaptation up-regulated the CKB at the protein level in both LV and RV. The CKB mRNA level remained unchanged. The mtCKs transcript tended to fall in the LV and markedly decreased in the RV. The protein expression of the mtCKs significantly increased in the LV and tended to decrease in the RV (*Figure 32*).

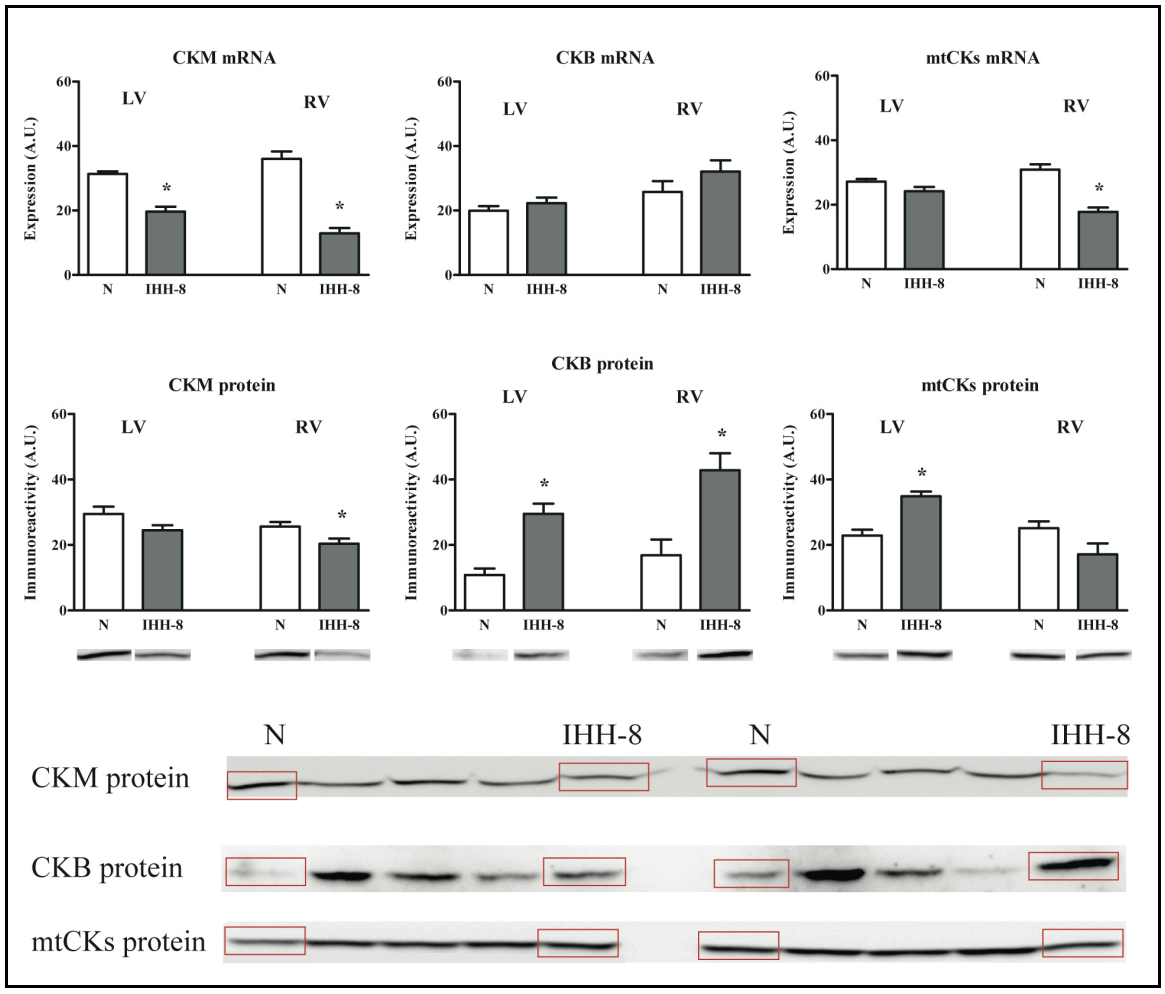

Figure 32: *The expression of CK isoforms in the left (LV) and right (RV) ventricles. The relative levels of CKM mRNA (top left), CKB mRNA (top middle), mtCKs mRNA (top right), CKM protein (middle left), CKB protein (middle middle), and mtCKs protein (middle right) are expressed as a percentage of total amount determined in the LV and RV from normoxic rats (N) and from rats adapted to the intermittent hypobaric hypoxia (IHH-8). The representative bands were cut out from the original gels containing also other experimental groups (bottom). Values are mean ± S.E.M. (n = 5). * P < 0.05 vs. N.*

The mRNA level of the HK1 did not change after the adaptation to the IHH-8, while the HK1 protein significantly increased in the LV and markedly decreased in the RV. The HK2 mRNA did not change, only tended to rise in the LV and decline in the RV. The protein expression of the HK2 substantially increased in the LV and had a tendency to increase in the RV (*Figure 33*).

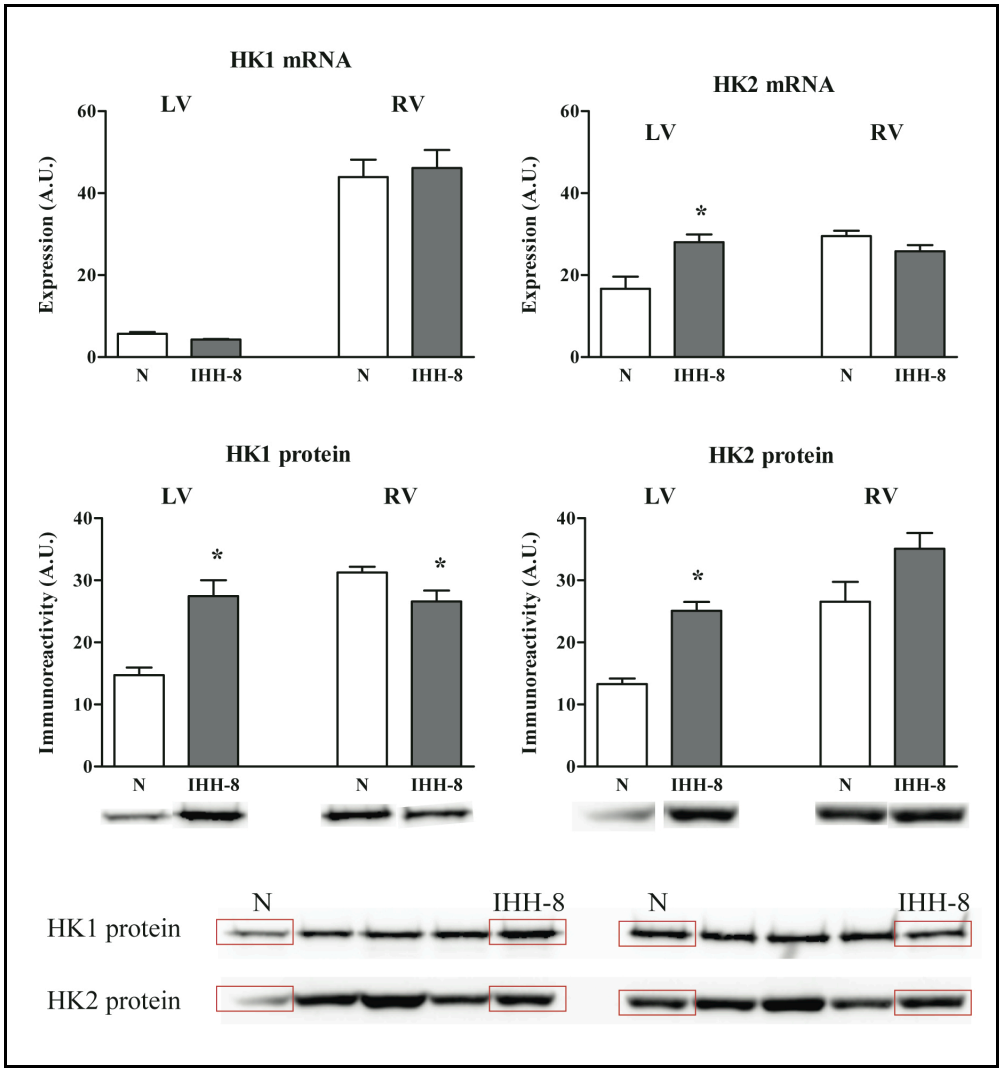

Figure 33: *The expression of HK1 and HK2 isoforms in the left (LV) and right (RV) ventricles. The relative levels of HK1 mRNA (top left), HK2 mRNA (top right), HK1 protein (middle left), and HK2 protein (middle right) are expressed as a percentage of total amount determined in the LV and RV from normoxic rats (N) and from rats adapted to the intermittent hypobaric hypoxia (IHH-8). The representative bands were cut out from the original gels containing also other experimental groups (bottom). Values are mean ± S.E.M. (n = 5). * P < 0.05 vs. N.*

The fluorescence analyses showed that the adaptation to the IHH-8 significantly increased the co-localization of the HK1 with mitochondria as well as the co-localization of the HK2 with mitochondria in the LV as well as in the RV (*Figure 34*). These observations were confirmed by the fractionation and WB method showing the elevated HK1 and HK2 protein levels in the mitochondrial fractions from the hypoxic LV and RV (*Figure 35*).

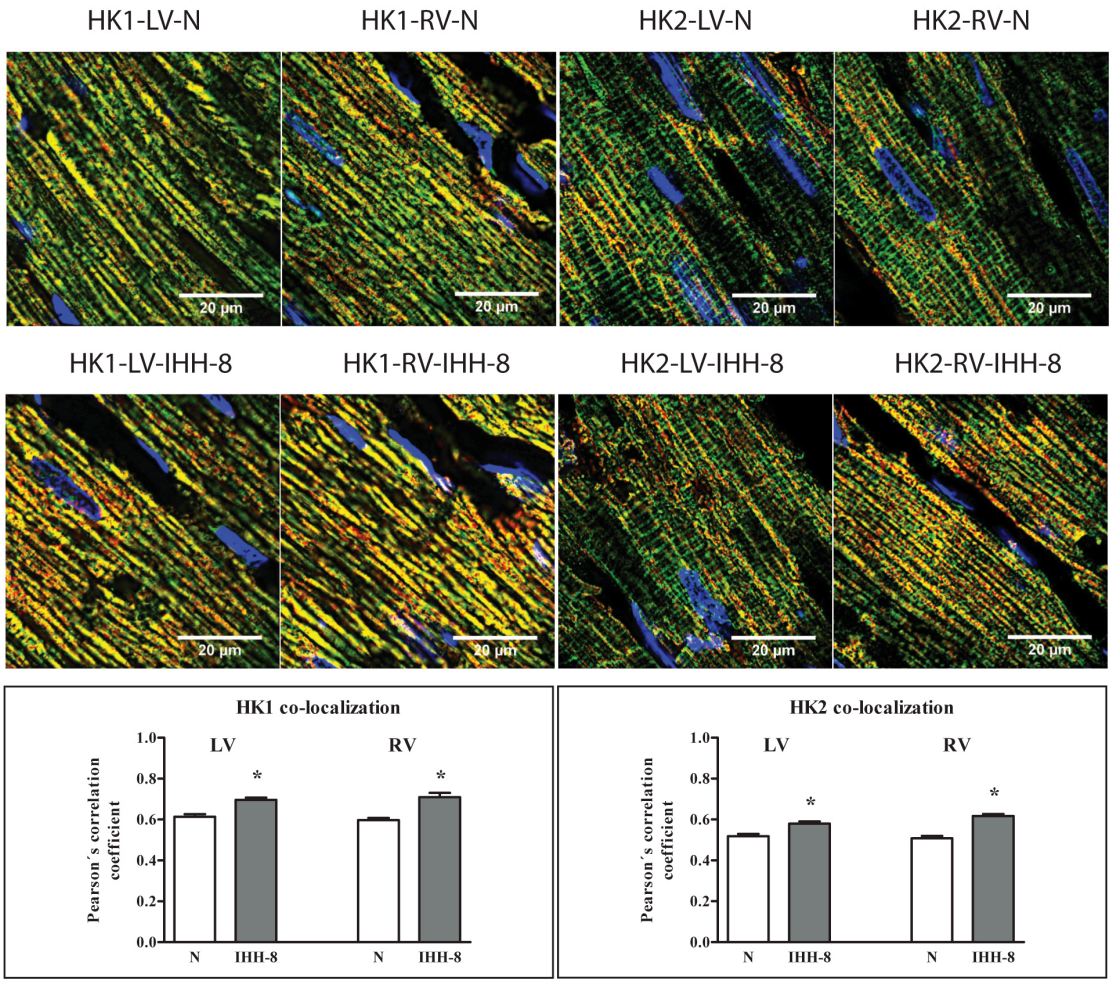

Figure 34: *The representative qualitative images of transversal cross cryosections showing the co-localization of the HK1 and HK2 with mitochondria in the myocardial left (LV) and right (RV) ventricles from normoxic rats (N) and from rats adapted to the intermittent hypobaric hypoxia (IHH-8) (top and middle). The green color represents the specific HK1 or HK2 staining and the blue color indicates the nuclear 4',6-diamidino-2-phenylindole (DAPI) staining. The red color represents the distribution of the mitochondrial compartments (I-V complexes). The increase in yellow-orange color indicates an increased co-localization of the HK1 and HK2 with mitochondria expressed as the Pearson´s correlation coefficients (bottom). The scale bar represents 20 µm. Values are mean ± S.E.M. (n = 6). * P < 0.05 vs. N.*

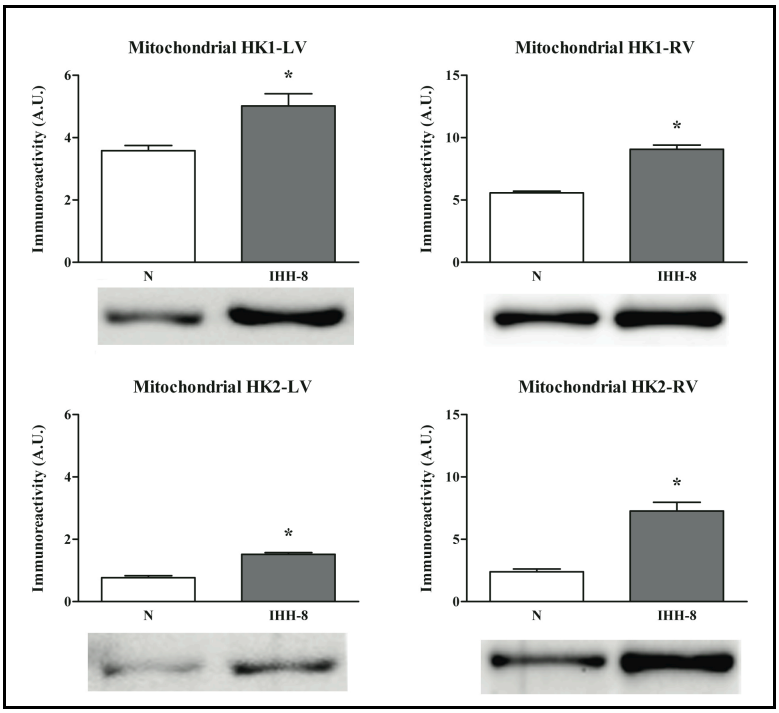

Figure 35: *The protein levels of the HK1 and HK2 in the mitochondrial fraction from the left (LV) and right (RV) ventricles. The relative levels of HK1 protein (top) and HK2 protein (bottom) are expressed as a percentage of total amount determined in the LV and RV from normoxic rats (N) and from rats adapted to the intermittent hypobaric hypoxia (IHH-8). Values are mean ± S.E.M. (n = 5). * P < 0.05 vs. N.*

6.3 Conclusion

The present study showed the increased CK activity, which is in line with the up-regulation of mtCKs and CKB proteins, indicating an elevated activity of PCr/CK system, which is responsible for maintaining energy homeostasis and ADP recycling in mitochondria. The up-regulation of HK1 and HK2 proteins and the total HK activity in the LV may be related to the protective metabolic mechanisms of hypobaric hypoxia enhancing the oxidative phosphorylation and consequently preventing oxidative stress. In addition, the higher HK1 as well as HK2 co-localization with mitochondria may suggest the activation of survival pathway in the heart of rats adapted to the severe intermittent hypobaric hypoxia.

7. DISCUSSION

7.1 Study 1: The comparison of the LV and RV under normoxia

The present study demonstrated a significantly higher expression of HK1 and HK2 isoforms at mRNA as well as protein level in the RV as compared to the LV. These findings were confirmed by immunofluorescence staining and fluorescence microscopy analyses, which revealed substantially higher fluorescence signals of both HKs in the RV than in the LV. To this author's knowledge, no data have been published to date comparing the expression and subcellular localization of HK isoforms in the RV and LV. The higher content of HK isoforms in the RV may suggest a higher activity of aerobic glycolytic metabolism in this ventricle, which can enhance oxidative phosphorylation and consequently attenuate oxidative stress.

7.1.1 Differences in energy metabolism between ventricles

The present results are supported by recent findings regarding the higher expression of the IGF1 in the RV than in the LV (Drake et al., 2011). The IGF1 regulates the glucose transport into the cells, glucose utilization and promotes glycolysis, which can be reflected in the increased expression of glycolytic enzymes, such as the HK (Cheng et al., 2000; Bondy and Cheng, 2004). It has been observed that the IGF1 induced the HK gene expression in a concentration and time dependent manner in two cancer cell lines (Sebastian and Kenkare, 1997). The IGF1 also plays a role in the cardiomyocyte growth and controls cell differentiation and apoptosis (Takeda et al., 2010). Besides that, the RV shows a lower expression of both fatty acid binding protein isoforms and a higher expression of glycogen debranching enzyme (Phillips et al., 2011), which may favor a higher glucose utilization in the RV. The activity of glucose metabolism is associated with the expression of contractile proteins, such as MyHCα and MyHCβ. MyHCα possesses a higher ATPase activity leading to a faster contraction, whereas MyHCβ with a lower ATPase activity is associated with a slower contraction (Ebrecht et al., 1982; Pope et al., 1980; Schwartz et al., 1982). Interestingly, a lower expression of MyHCβ and a higher expression of MyHCα has been observed in the RV

as compared to the LV (Brooks et al., 1987; Sharma et al., 2003), indicating that the RV exhibits a faster muscle phenotype. Indeed, the shortening velocity of RV muscle is greater than that of the LV (Brooks et al., 1987).

The faster contraction requires a faster transport of the ATP provided by the CK. Although, mRNA levels of all CK isoforms were significantly higher in the RV than in the LV, no differences at protein levels of CK isoforms were found between ventricles, except for the CKB protein, which tended to be higher in the RV than in the LV. Similarly, total CK activity did not differ between ventricles. This may suggest a higher contribution of glycolysis to phosphotransfer network in the RV as compared to the LV.

7.1.2 Coronary blood flow differences between ventricles

Increased metabolism rate requires an enhanced oxygen delivery, which is provided by an increased coronary blood flow. It has been shown that the collagen concentration in the adult RV is higher than that in the LV (Caspari et al., 1975b; a; Ostadal et al., 1978). This higher collagen concentration in the RV is associated with a higher static elasticity of right myocardium (Cappelli et al., 1984) and can reflect the distribution of coronary vasculature (Buccino et al., 1969). The number of capillaries per mm^2 is significantly greater in the RV than in the LV (Henquell and Honig, 1976). Experimental studies dealing with amino acid incorporation into the heart have also found differences between the RV and LV. The incorporation of ^{14}C-Proline and ^{14}C-Lysine into the RV was significantly higher than into the LV (Ostadal et al., 1978; Schreiber et al., 1966). The authors assumed that increased incorporation into the RV might be due to a more rapid turnover or greater coronary perfusion per unit of muscle mass (Ostadal et al., 1978). The higher coronary blood flow in the RV can be also due to a lesser systolic compression of the arteries, as compared to the LV, so the coronary blood flow is more continuous. Recently, Drake et al. (2011) have reported that the gene encoding the nuclear receptor subfamily 2, group F, member 2 (NR2F2) protein was found to be expressed 2-fold higher in the normal RV than in the LV (Drake et al., 2011). The NR2F2 is required for angiogenesis during heart development (Pereira et al., 1999) and it might thus correspond to the increased vascularization in the RV observed by Henquell and Honig (1976).

7.1.3 The interaction of HK isoforms with mitochondria

Hexokinase represents an essential glycolytic enzyme playing an important role in survival pathways. Several reports have shown that an increased expression of HK is associated with an improved resistance of cells, including cardiomyocytes, against ischemic injury (Zuurbier et al., 2009) and apoptosis (Ahmad et al., 2002; Azoulay-Zohar et al., 2004; Chiara et al., 2008; Miyamoto et al., 2008; Pastorino et al., 2002; Sun et al., 2008). The anti-apoptotic importance of HK lies to a high degree in the association of this enzyme with mitochondria. However, neither HK1 nor HK2 co-localization with mitochondria differed between ventricles in the present study. On the other hand, the immunofluorescence analyses showed that the co-localization of HK1 with mitochondria is greater than that of the HK2 in both ventricles. Southworth et al. (2007) have performed a very detailed study focused on the distribution of HK in LV cardiomyocytes using electron microscopy. They have found that in the heart under resting conditions, HK1 is associated with mitochondria to a much higher extent (10-fold) than HK2 and that the binding of each enzyme isoform to mitochondria is regulated differently. Interestingly, under ischemic conditions or increased level of insulin (Southworth et al., 2007) as well as under increased concentration of glucose (John et al., 2011), the association of HK2 with mitochondria increased 5-fold, while that of HK1 only 2-fold (Southworth et al., 2007).

Recently, it has been reported that fully activated phospho-AKT stimulated HK2 translocation to the outer mitochondrial membrane (Miyamoto et al., 2008; Roberts et al., 2013), where HK2 inhibited the binding of the pro-apoptotic BAX protein (Pastorino et al., 2002) and the opening of the MPT pore (Azoulay-Zohar et al., 2004; Beutner et al., 1998). Full activation of AKT requires phosphorylation at both Ser and Thr residues (Alessi et al., 1996). In the present study, the expression of phospho-Ser-AKT as well as phospho-Thr-AKT was significantly higher in the RV than in the LV. In addition, both ratios of phospho-Ser-AKT/non-phosphorylated AKT and phospho-Thr-AKT/non-phosphorylated AKT were markedly higher in the RV than in the LV. Nevertheless, the higher level of activated AKT in the RV did not lead to an increased co-localization of HK2 with the mitochondria. These findings suggest that AKT activation is a necessary but not a sufficient condition for the enhancement of the interaction of HK2 with mitochondria and that yet another mechanism

may be involved, initiated by a complex physiological processes such those associated with an increased concentrations of glucose or insulin, for which the increased binding of HK2 to mitochondria was described (John et al., 2011; Southworth et al., 2007).

7.1.4 The HK enzyme activity in the LV and RV

The increased binding of HK with mitochondria generally results in an increased HK activity because of the reduction of G-6-P inhibitory effect (Parra et al., 1997). Most studies dealing with HK in the heart focused only on the enzyme activity in homogenate. Although higher activity of HK in the RV as compared to the LV was found in young rats (Bass et al., 2001; De Tata et al., 1988), other studies did not detect any significant right-to-left ventricular difference (Bass et al., 1993; Daneshrad et al., 2000; Rumsey et al., 1999). This is in accord with the results of the present study, supporting the co-localization findings, too. In contrast, the activity does not correlate with the higher expression of HK1 and HK2 observed in the RV as compared to the LV. This apparent discordance may be explained by the fact that comparable concentrations of both HK isoforms bound to mitochondria were detected in both ventricles. The increased total concentration of HK in the RV as compared to the LV can be due to higher levels of cytosolic forms of the enzyme. Taking into account that HK associated with mitochondria contributes principally to a higher enzyme activity, the increased levels of cytosolic HK in the RV may not necessarily affect the total enzyme activity. Moreover, changes in the total HK activity correlate with specific changes in the activity of the cytosolic isoform HK2 rather than with the activity of HK1, which is predominantly bound to mitochondria (Riddle et al., 2000).

When comparing the expression and the activity of HK, it is important to keep in mind that standard WB analysis under denaturing conditions only allows the detection of the monomeric form of the enzyme, while the dimers or tetramers may substantially differ in their activity (Hoggett and Kellett, 1992). Post-translational modifications such as sumoylation (Aslanukov et al., 2006) and ubiquitinylation (Magnani et al., 1994) can affect the enzyme activity of HK and cause its degradation. Hence, the enzyme activity need not be directly proportional to the relative amount of HK determined by WB. Moreover, tissue homogenization could disrupt many of these levels of regulation, including detaching the HK

from mitochondria. This means that *in vitro* measurements of HK activity may differ from the real *in vivo* tissue activities.

The mechanisms underlying the enhancement of enzyme activity induced by HK interaction with the mitochondria include conformational changes of the enzyme molecule and Pi competition with G-6-P at its binding site. The HK2 forms dimers in the cytosol, while the interaction of HK2 with mitochondria requires HK in a tetrameric form, as well as the HK1-mitochondria interaction (Mulichak et al., 1998; Wilson, 1995). The HK monomer-dimer-tetramer transitions and their interactions with mitochondria may further affect the conformational state of the monomers and thus change their substrate-binding affinity resulting in the increased activity of the whole complex. The question then arises how oligomerization of HK can change the enzyme activity of monomers. Another question is whether the increased enzyme activity due to its binding to the mitochondria is caused by product channeling (Gregor et al., 2003), i.e., by a shift of the equilibrium to the right, or through an increased number of active monomers formed by conformational changes induced by the interaction itself. Based on the different functional properties between HK1 and HK2, the different contribution of each HK isoform to the total HK activity can be expected.

7.2 Study 2: The effect of the normobaric hypoxia and I/R insult on the CK and HK enzymes

The present study showed significantly increased enzyme activities of CK and HK and expression of mtCKs, CKB and both HK isoforms in the LV induced by adaptation to the moderate normobaric hypoxia. The up-regulation of the mitochondrial enzymes and their activities may indicate a higher stimulation of the respiratory chain *via* ADP recycling, which can reduce ROS and thus help to prevent oxidative stress.

7.2.1 The CK enzyme activity in the LV

The CK molecule is known to be very susceptible to oxidative stress, which causes a dissociation of SH bonds between cystein residues in each monomer and thus affects the CK catalytic activity (Konorev et al., 1998; Koufen et al., 1999; Mekhfi et al., 1996; Wendt et al.,

2003). The significant increase of the total CK activity in the LV of rats adapted to protective regimens of normobaric hypoxia (CNH, INH-8) suggests that the cardiac energy metabolism responses physiologically under these conditions, which are not associated with a severe oxidative stress. This is likely due to an increased capacity of the antioxidant system, which is responsible for the control of the ROS over-production. This is supported by the finding of an increased mRNA expression of major antioxidant enzymes (Kasparova et al., data in preparation). In addition, the total CK activity did not change in the LV during an acute I/R insult suggesting that the adaptation to CNH regimen preserves the CK function and possibly preserves the octamer-dimer transition of the mtCKs. Adaptation to normobaric hypoxia could thus have similar protective effects as ischemic preconditioning (Laclau et al., 2001).

7.2.2 The CK expression in the LV

The total CK activity represents all CK isoforms at the protein level. The adaptation to CNH and INH-8 regimens significantly increased expression of mtCKs and CKB isoforms, while did not change the CKM expression. In view of the fact that CKB represents a minor isoform in the mature heart (*Table 4*), it can be assumed that the increase of the total CK activity under hypoxic conditions can be attributed mainly to mtCKs.

Table 4: *The mRNA and protein levels of CK isoforms in the normoxic LV and RV expressed as percentage of total.*

CK isoform	protein %		mRNA %	
	LV	RV	LV	RV
mtCKs	37.44	43.88	48.12	47.96
CKM	54.48	49.48	51.58	51.69
CKB	8.07	6.64	0.30	0.34

The mRNA level of CKB is by two orders of magnitude lower than that of mtCKs and CKM in the normoxic LV. The mtCKs and CKM mRNA/mRNA ratios are close to 1:1 in the LV suggesting equilibrium between PCr production in mitochondria and its utilization close to ATPases mediated by CKM under physiological conditions. It seems that increased expression of mtCKs under hypoxic conditions may result in an elevated PCr production in mitochondria leading to the increased availability of ADP for ATP synthase. The accelerated PCr synthesis has been already shown after adaptation to chronic hypoxia (Novel-Chate et al., 1995). Moreover, it has been reported that increased mtCK levels could help to sustain a high energy turnover, which may be beneficial under stress situations (Carter et al., 1995; Holtzman et al., 1998) and possibly protect cells from apoptosis (Kornacker et al., 2001). It appears that the up-regulation of mtCKs and increase of the total CK activity induced by adaptation to protective regimens of normobaric hypoxia can represent a compensatory mechanism engaged in improving oxidative energy metabolism. However, the question remains why mtCKs protein level and total CK activity also increased after adaptation to a non-protective regimen of normobaric hypoxia (INH-23). The CKB protein also tended to increase, but not significantly, and mRNA levels of both mtCKs and CKB remained unchanged. One of the possible explanations could be that the elevated total CK activity and mtCKs protein level mainly represent the adaptive mechanism of CK system to hypoxia, which is able to maintain adequate phosphotransfer homeostasis within cardiomyocytes. The cardioprotective phenotype of CNH and INH-8 regimens may therefore be related to the activation of other mechanisms, such as antioxidant system, other enzymes of energy metabolism, or pro-survival kinases.

From the literature data it is evident that the model of normobaric continuous hypoxia is not as frequent as the model of hypobaric intermittent hypoxia. There is only one study (Novel-Chate et al., 1998) which has used the same model and focused on CK. However, this study has reported opposite results showing a decrease of total CK and mtCKs activities after adaptation to normobaric (10% O_2) hypoxia for 3 weeks. However, it should be noted, that the authors used female rats and that significant differences may exist in the sensitivity of males and females to hypoxia (Ostadal et al., 1984b).

The regulation of mtCKs gene expression still remains unclear. It is known that mtCKs is regulated by the same transcription factors from the MyoD and MEF2 family of proteins as CKM (Qin et al., 1998). Nevertheless, the results showed a discordant trend in the expression

between two major isoforms mtCKs and CKM in the LV of all hypoxic groups, suggesting different regulatory mechanisms controlling the expression of these genes during hypoxia. One of the possible factors playing a role in a transcriptional regulation of mtCKs is the mitochondrial transcription factor A (mtTFA), which is activated under impaired mitochondrial energy supply and is involved in mitochondrial proliferation (Wiesner et al., 1999) stimulated also by chronic hypoxia (Nouette-Gaulain et al., 2005).

The up-regulation of CKB induced by normobaric hypoxia seems to be controlled at the transcriptional (Willis et al., 2005; Wu-Peng et al., 1992) as well as translational (Ch'ng et al., 1990) and post-translational (Chida et al., 1990; Hemmer et al., 1993) levels. The post-translational modification usually leads to phosphorylation of CKB mediated by kinases, such as PKC (Chida et al., 1990; Hemmer et al., 1993). The phosphorylated form of CKB has an increased affinity for PCr (Quest et al., 1990) allowing CKB to transfer the high-energy phosphate from PCr to ADP. This may provide a higher ATP supply for ATP-requiring processes at low PCr levels (Hemmer et al., 1993), which may likely occur also during hypoxia. Other potential candidate is the AMPK, which is activated under metabolic stress conditions (Emerling et al., 2009; Jing et al., 2008; Mungai et al., 2011), phosphorylates CKB and thus allows CKB to regulate the SERCA activity (Rios et al., 2014). The CKB is predominantly expressed during prenatal life and it is better adapted to stressful conditions (Mahadevan et al., 1984).

7.2.3 The CK expression and enzyme activity in the RV

Adaptation to chronic hypoxia has a beneficial effect on the LV as well as on the RV. Even the hypoxic RV is more tolerant of ischemia than the hypoxic LV. However, the hypoxia also causes a pulmonary hypertension leading to a pressure overload and hypertrophy of the RV (Baker et al., 1997). The degree of the hypertrophy correlates with the intensity and duration of the hypoxic exposure (Bonnet et al., 2001). In the present study, the relative RV weight increased by ~ 86% at CNH, 69% at INH-23, and only 19% at INH-8 regimen. No changes in the LV weight were observed (*Table 1*). The LV is not exposed to increased afterload during the moderate hypoxia. Its weight therefore remains unchanged and it may increase only after prolonged exposure to the severe intermittent hypoxia (Cazorla et al., 2006;

Kolar et al., 2007; La Padula and Costa, 2005; Neckar et al., 2005; Widimsky et al., 1973), which is associated with moderate systemic hypertension. Hence, chronic hypoxia acts differently on the LV and RV energy metabolism (Daneshrad et al., 2000; Cai et al., 2010; Letout et al., 2005; Nouette-Gaulain et al., 2005; Novel-Chate et al., 1998; Pissarek et al., 1997; Rumsey et al., 1999). In the present study, the observed significant increase of the total CK activity in the RV at INH-8 regimen compared to CNH and INH-23 regimens may be possibly related to a lower oxidative stress. The protein level of mCKs was slightly higher in the normoxic RV compared to the LV and it remained unchanged after the adaptation to all hypoxic regimens, which can be possibly explained by the RV hypertrophy masking its potential increase. On the other hand, the same expression profile of mtCKs was found in the RV also at the mRNA level, so the question is whether it is due to a hypertrophy or not. This could also be associated with the mitochondrial biogenesis activating in the hypoxic LV and not in the hypoxic RV. The cytosolic CK isoforms showed a similar profile at mRNA as well as protein levels in both ventricles suggesting a direct effect of hypoxia on these isoforms. The compensation increase of CKB isoform induced by the adaptation to hypoxia has already been described (Letout et al., 2005; Pissarek et al., 1997). The CKB increase at protein level was markedly higher than that at mRNA level, which suggested the post-transcriptional or post-translational regulation (Shen et al., 2003).

7.2.4 The HK expression and enzyme activity in the LV

Adaptation to chronic hypoxia causes a shift from fatty acid oxidation to a glycolysis (Holden et al., 1995), which was also supported by the obtained results. The present study demonstrated that HK1 as well as HK2 protein level significantly increased in the LV of all hypoxic groups. The mRNA level of HK2 also markedly increased, which is in agreement with results obtained by Rumsey et al. (1999) using the same model of hypoxia. However, the HK1 mRNA remained unchanged, which is not in line with Rumsey et al. (1999), who have shown a significantly increased HK1 mRNA in both ventricles (Rumsey et al., 1999). Interestingly, different responses of HK1 at mRNA and protein levels were observed also in the human lung cell line A549 adapted to hypoxia (Riddle et al., 2000), which may indicate a higher activity of translational machinery. While the promotor region and potential

transcription factors of HK1 have been described (Chapter 1.3), the mechanisms regulating HK1 translation are still not known. These mechanisms may include the activation of initiation factors and transfer RNA, or inhibition of translational repressor proteins. The regulation of the HK2 gene expression has been studied more intensively. One of the potential HK2 regulators under hypoxia could be the transcriptional factor HIF1 (Riddle et al., 2000). The HIF1 is also activated *via* PI3K/AKT pathway (Gordan and Simon, 2007; Semenza, 2003), the same pathway, which activates HK2 expression (Osawa et al., 1996b). Adaptation to hypoxia leads to an increased level of catecholamines and cAMP, which may activate putative response elements in the HK2 promotor region (Mathupala et al., 1995; Rempel et al., 1996; Osawa et al., 1995, 1996a). Another important regulator is AMPK (Stoppani et al., 2002) also stimulating the total HK activity (Holmes et al., 1999), which in the present study increased in the LV after adaptation to all hypoxic regimens. The AMPK also inhibits the CKM activity (Ponticos et al., 1998) and, as mentioned earlier, it positively regulates CKB isoform (Rios et al., 2014). In addition to post-translational modification mediated by kinases, the HK activity could be also modulated by its substrate and product concentrations. It is known that hypoxia increases the glucose flux into cardiomyocytes leading to an elevation of the HK activity. This produces G-6-P, which is immediately metabolized in glycolysis and thus cannot reversely inhibit the HK activity (Chapter 1.5.2.3).

7.2.5 The HK interaction with mitochondria

The enhancement of the HK activity could be achieved by its greater association with mitochondria. It has been shown that AKT kinase phosphorylates HK2 and stimulates its translocation to the mitochondria (Miyamoto et al., 2008; Roberts et al., 2013). However, this mechanism does not seem to play a role in the stimulation of the HK activity under present experimental conditions, because no increase in mitochondrial co-localization of HK isoforms was observed after adaptation to normobaric hypoxia. Nevertheless, it should be mentioned that the present HK assay allowed determining the maximal specific activity of both HK isoforms independently of the localization of the enzyme molecules. Therefore, the disproportionately smaller increase of HK activity as compared to the higher increase of HK expressions may rather indicate the effect of post-translational modifications than changes in

interactions of HK with mitochondria. In addition, the conformational changes leading to a higher affinity of the enzyme for substrate do not have to affect the interaction of the enzyme with subcellular structures. Although the HK1 and HK2 co-localizations with mitochondria did not increase, they also did not decrease after adaptation to normobaric hypoxia. Hence, it seems that the present protective CNH regimen, similarly as ischemic preconditioning, could stabilize the binding of HK with mitochondria. Recently, Pasdois et al. (2013) have reported that ischemic preconditioning reduces HK2 loss from mitochondrial membrane during prolonged I/R insult, thereby reducing cytochrome *c* release, oxidative stress, and probability of the MPT pore opening (Pasdois et al., 2013).

7.2.6 The HK expression and enzyme activity after I/R insult

The present study also involves I/R experiments on isolated normoxic hearts and hearts from rats adapted to CNH regimen that showed a significantly increased HK activity in the hypoxic LV after I/R insult as compared to non-ischemic ventricles. Moreover, the Bcl-2/BAX ratio was markedly higher in hypoxic LVs subjected to the I/R insult than in normoxic ventricle. These results may suggest that adaptation to CNH regimen can preserve the binding of HK with mitochondria during the I/R injury, thereby reducing the probability of BAX binding into mitochondria to activate apoptosis. The protein level of HK1 remained unchanged in hypoxic LVs after the I/R insult, although a tendency to its increase was observed in LVs adapted to hypoxia as compared to the normoxic ones. Similar trend was also observed in the total HK activity and HK2 protein level. In addition, HK2 protein tended to increase more after ischemia in the hypoxic LV than in the normoxic LV, which may reflect the contribution of this HK isoform to ischemia-resistant phenotype of CNH hearts.

7.2.7 The HK expression and enzyme activity in the RV

Regarding the HK1 and HK2 expressions in the RV, there were not any differences between experimental groups. Only adaptation to INH-8 regimen significantly increased HK2 mRNA in the RV. HK1 and HK2 protein levels were higher in the RV compared to the LV under normoxic condition. These findings were already confirmed and discussed previously

(Chapter 7.1). It was also observed a markedly higher mRNA level of HK1 in the RV as compared to the LV in all hypoxic regimens, which could be associated with the development of the RV hypertrophy. The total HK activity significantly increased in the RV of rats adapted to all hypoxic regimens. The marked differences between RV and LV were found in CNH and INH-23 groups, which can be explained by differences in the oxidative stress between these two hypoxic regimens. The present data are in line with other studies focused on the effect of normobaric hypoxia on the HK activity (Daneshrad et al., 2000; Rumsey et al., 1999).

7.3 Study 3: The effect of the hypobaric hypoxia on the CK and HK enzymes

7.3.1 The comparison of CK expression and enzyme activity with the INH-8 regimen

The present study demonstrated the significantly increased total CK activity and elevated protein levels of mtCKs and CKB in the LV of rats adapted to IHH-8 regimen. The same results have been also obtained after adaptation of rats to a less severe model of intermittent normobaric hypoxia, INH-8 (Chapter 5.2.1). These findings showed that a stronger hypoxic stimulus, which is just IHH-8 adaptation, still maintains active PCr/CK system for optimal energy homeostasis and ADP recycling in mitochondria. In contrast, mtCKs and CKB mRNA levels remained unchanged in the LV of IHH-8 rats as compared to those adapted to milder normobaric hypoxic regimens, where mRNA levels substantially increased or tended to increase (Chapter 5.2.1). These observations may suggest the involvement of different transcriptional and post-transcriptional regulatory mechanisms. Moreover, the LV has to be able to maintain contractile function. Therefore most ATP is used for muscle contraction, whereas transcription processes are inhibited. With respect to RV, changes in CKB expression were similar, but the expression of mtCKs and CKM significantly decreased after IHH-8 as compared to INH-8 regimen. These progressive changes observed in the IHH-8 group can be due to a greater RV afterload imposed by pulmonary hypertension.

7.3.2 The comparison of the CK function with the published data

Comparing present data with other studies is very complicated due to different models and adaptation regimens of hypoxia. In addition, other studies have determined the enzyme activity of CK isoforms, while the present study demonstrated the expression of CK isoforms. For example, Letout et al. (2005) have reported similar results related to the total CK activity and CKB and mtCKs activities in the LV, but they have observed an opposite results for CKM activity in the LV and for total CK activity and CK isoforms activities in the RV (Letout et al., 2005). This can be explained by the fact that the authors had adapted rats to much lower degree of hypoxia compared to the present study, which might result in distinct cellular responses and the degree of RV pressure overload. Moreover, they had studied the effect of hypoxia on Sprague-Dawley rats, as compared to the present study on Wistar rats, which also may have affected the results. Another study has reported a decrease of the total CK activity and mtCKs activity in the LV of rats adapted to hypobaric hypoxia (Pissarek et al., 1997). In this case, the authors used a model of continuous hypobaric hypoxia with only 30-45 min of reoxygenation per day, which could be associated with a greater oxidative stress than the present model of intermittent hypobaric hypoxia. The increased total CK activity and elevated protein levels of mtCKs and CKB in the LV may result from post-translational modifications.

7.3.3 The comparison of HK expression and enzyme activity with the INH-8 regimen

As expected, the total HK activity also markedly increased in the LV of rats adapted to IHH-8 regimen, which is in line with elevated HK1 and HK2 protein levels. The HK2 mRNA only tended to increase and HK1 mRNA remained unchanged. The discrepancy between HK1 mRNA and protein expression is discussed in the Chapter 7.2 and the differences between normoxic LV and RV in the Chapter 7.1. The up-regulation of HK1 and HK2 proteins and the increased total HK activity in the LV may be related to the protective metabolic mechanisms of hypobaric hypoxia enhancing the oxidative phosphorylation. In addition, HK1 and HK2 co-localizations with mitochondria significantly increased after adaptation to IHH-8 regimen, which may indicate the activation of survival pathways in cardiomyocytes.

The present model of hypobaric hypoxia simulating an altitude of 7000 m seems to be quite extreme in terms of adaptive potential and may already result in some maladaptive changes. It has been shown that IHH-8 regimen, unlike less severe conditions of chronic hypoxia, may increase systemic blood pressure causing mild LV hypertrophy, fibrosis, and diastolic dysfunction in addition to focal micro-necrosis (Boussuges et al., 2000; Kjaergaard et al., 2006; Kolar et al., 1989; Ostadal et al., 1981; Urbanova et al., 1977; Widimsky et al., 1980). Nevertheless, the LV myocardium of rats adapted to IHH-8 regimen still retains the improved resistance to the I/R injury (Kolar et al., 2007; Neckar et al., 2002a). The specific protective effect of HK lies in its increased binding with mitochondria, which results in the inhibition of apoptosis. Therefore, it seems that adaptation to IHH-8 is associated with ROS-dependent signaling, which leads to the activation of pro-survival pathways that may possibly involve the HK binding to mitochondria. One of the important pro-survival pathways resulting in the stimulation of HK association with mitochondria is the PI3K/AKT pathway, because it has been found that activated AKT stimulated HK2 translocation into mitochondria (Miyamoto et al., 2008; Roberts et al., 2013). It has been reported that IHH-8 regimen induced the activation of AKT mediated by its phosphorylation in the LV but not in the RV (Strniskova et al., 2006). However, the present results showed substantially increased HK1 and HK2 co-localizations with mitochondria also in the RV. This indicates that the increased co-localization of HK isoforms with mitochondria is directly induced by hypoxia in both ventricles. This supports the view that the stimulation of HK co-localization with mitochondria is associated with a distinct pathway independent on the AKT activation.

7.4 Conclusion

The results of the present study indicate that the normoxic RV has a higher activity of aerobic glycolytic metabolism and may be able to respond faster and more powerfully to stressful stimuli than the LV. Furthermore, the study demonstrates that adaptation to normobaric hypoxia as well as to more severe hypobaric hypoxia increases mtCKs, CKB, HK1, and HK2 expressions and total CK and HK activities in the LV, which reflects the adaptation of energy metabolism to a decreased tissue oxygen concentration regardless the model and type of hypoxia. These results also suggest that CK and HK co-operate together to maintain energy

homeostasis and provide an adequate ATP production and transfer in the LV under hypoxia. Adaptation to hypoxia evokes apparently dissimilar metabolic responses in the LV and RV, which could be related to RV hypertrophy. Based on the results from co-localization experiments, it can be assumed that the cardioprotective role of HK lies in its interaction with mitochondria, which depends on the degree of oxidative stress. In particular, HK may play a crucial role in the caridoprotective mechanism induced by severe hypobaric hypoxia, which represents an extreme model of adaptation.

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